

LIPOIDS AND BLOOD PLATELETS

WITH REFERENCE TO BLOOD COAGULATION
AND THE HEMORRHAGIC DISEASES

By

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Im Tatsächlichen grosster Pragnanz

Im Hypothetischen ausserste Vorsicht (Wohlisch 1943)

When theory fancy free
Bids hither yon & the way
Calm fact exhorteth thee
Consider tyro' Stay (Orig)

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EDITORIAL NOTE

The author adopts the American spelling in the text

Bibliography:

- (1) References are given in the text by numerals in square brackets corresponding to alphabetized bibliography in Appendix III
- (2) Titles of journals are abbreviated in accordance with the widely used List of periodicals abstracted by Chemical Abstracts American Chemical Society (Columbus Ohio) 1951
- (3) Abstracts (abstr) and 'personal communications' are indicated
- (4) Authors are listed alphabetically In case of multiple authorship the second and subsequent authors' names follow in alphabetical sequence
- (5) Diphthongs are replaced by separate letters Modified letters (accent etc) when not anglicized (e g ue German u) in the original reference appear in the usual alphabet order but include the modification
- (6) Hyphenated and compound names are listed in alphabetical order under initial letter of surname including De Le Mac Mc Van Von

Author

ABSTRACT

By specifically analyzing for the various active principles of plasma platelets tissues and their fractions much new information has been obtained concerning the role of lipids and platelets in blood coagulation and in the hemostatic mechanisms in health and disease. Analyzed components are studied in artificial clotting systems especially a two stage thrombin forming system. Some 86 cases of bleeding disorders 32 new born normal infants and their mothers and many normal adult bloods have been analyzed with respect to components of the clotting and hemostatic functions.

The detailed considerations embodied in the dissertation are encompassed under the following heads:

- 1) the importance of certain lipids especially cephalin
- 2) the normal need for platelets in plasma clotting
- 3) the particular significance of a platelet component which has many analogies to cephalin in the thromboplastic system
- 4) potentiation of the thromboplastic actions of cephalin of platelets and of tissue thromboplastin (to some extent) by a variety of experimental additives. Part of this may be explained as a 'thromboplastin generation' through co participation of certain plasmatic components (antihemophilic globulin PTC etc). Part however may be the result of certain proteolytic enzymes particularly trypsin 'disaggregating' lipoproteins and thus rendering their phospholipid (and sometimes calcium) available for participation in the clotting reactions.
- 5) possible Ca containing and lipid containing 'intermediates' in the thrombin forming reactions
- 6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as thrombocytes megakaryocytes and stomatolytic erythrocytes
- 7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanisms
- 8) the occurrence of many clinical disorders due to deficiency of platelet functions. Thrombocytopenias denote deficient numbers (counts and total bulk in body). Thrombocytopathias are deficiencies of specific platelet components e.g. thromboplastic factor accelerator vasoconstrictor (5 hydroxytryptamine) or retractor factor. Such deficiencies can be clinically significant even when the platelet count is normal. Bleeding in leukemias uremias etc. may often be accounted for in these terms.
- 9) the nature and modes of action of heparin and other 'antithromboplastic' inhibitors and of some antiproteases in relation to the mechanisms discussed
- 10) the 'cephalin availability theory' of the author as a useful working hypothesis to explain the importance of the natural thromboplastic phospholipid. Lipid release from platelet tissue or possibly plasma sources may very well be the long obscure 'trigger mechanism' which initiates blood coagulation.
- 11) The supplement reports 11 new experiments in the period 1956-1959. The versatile two stage method has been modified for the quantitative study of each individual factor in the blood-clotting system and adds some

new data concerning thromboplastic mechanisms. These data contribute further support for the main thesis. It is suggested that the term 'prothromboplastic' be used to designate the phosphatide (probably cephalin) which in conjunction with calcium ions and a diverse (experimentally) group of accessory factors constitutes the mechanism for activation of prothrombin to thrombin. 'Availability of the lipid as a trigger mechanism' and its dynamic role in these activator mechanisms seem best explained in terms of a 'phospholipid transfer' hypothesis.

CONTENTS

Page

PART I BACKGROUND

FOREWORD selected reviews; changing attitudes of Science 3

CHANGING CONCEPTS IN THE CLOTTING FIELD 4

A HISTORICAL HIGHLIGHTS 4

- 1 Early Origins: Hewson Denis Buchanan 4
- 2 The Dorpat School 5
- 3 The Work of Hammarsten and Wooldridge 7
- 4 The Calcium Factor 7
- 5 The Morawitz Fuld Theory 8
- 6 The Heterodoxy of Nolf 8
- 7 The Howell Theory 9
- 8 Mellanby 12
- 9 Mills Practice vs Precept 14

B MODERN VIEWS ON BLOOD CLOTTING 15

- 1 Skeleton Scheme of Clotting Reactions 15
- 2 Extended Scheme of Clotting and Hemostatic Mechanisms 16
- 3 The Thrombin Fibrinogen Reaction 16
 - a) Fibrin yields 17
 - b) Clotting times 18
 - (1) Concentration of thrombin 18
 - (2) Concentration of fibrinogen 18
 - (3) Temperature 18
 - (4) pH 19
 - (5) Ionic strength 19
 - (6) Specific ion effects 19
 - (7) Reversible inhibitors 19
 - (8) 'Fibrinoplastic' colloids 19
 - (9) Partial denaturation of fibrinogen 19
 - (10) Adsorptive removal of thrombin 20
 - c) Nature of fibrin formation 20
 - d) Thrombin 21
 - e) Other coagulants etc 22
- 4 Conversion of Prothrombin to Thrombin 22
 - a) Purity of prothrombin preparations 22
 - b) Nature of prothrombin 23
 - c) Biophysics of thrombin formation 23
 - (1) 'Biothrombin' 23
 - (2) 'Citrate thrombin' 23
 - (3) 'Trypsin thrombin' 24
 - d) 'Prothrombinogen' (?) 24
 - e) Calcium in relation to the conversion of prothrombin into (bio)thrombin 24
 - f) Thromboplastin and the activation of prothrombin 25
 - g) 'Thromboplastic enzyme' 25
 - h) 'Accelerator' factors in prothrombin conversion 26

i) 'Converter' factors in prothrombin activation	26
j) Plasma factors in thromboplastin generation	27
(1) Hemophilia and related problems	27
(a) Hemophilia A	27
(b) PTC-deficiency (Hemophilia B)	28
(c) PTA-deficiency (Hemophilia C)	29
(d) 'Fourth factor' deficiency (Hemophilia D?)	29
(2) 'Hageman factor'	29
(3) 'Factor X'	29
5 Inhibitor Problems	29
a) Inactivators of thrombin	29
b) Heparin	30
c) Heparin co factor (heparin complement)	31
d) Specific inhibitors of individual clotting factors	31
(1) Antiplatelet factors	31
(2) Anti-AHF and anti PTC	31
(3) Anti(pro)convertin (factor VII inhibitor)	32
(4) Anti(pro)accelerin (factor V inhibitor)	32
(5) Antithromboplastin A	32
Antithromboplastin B	32
e) Summary of the inhibitor problems and their clinical significance	33

BLOOD PLATELETS 33

A HISTORICAL 33

1 Platelets in the Blood Under Normal and Thrombotic Conditions	35
2 Viscous Metamorphosis and Platelet Adhesiveness	35
3 Platelet 'Agglutination' (Normal)	36
4 Platelet Antisera Platelet Groups and the Thrombocytopenia Problem	37
5 In Vitro Alterations of Blood Platelets	38

B PLATELET FUNCTIONS 38

1 Relation of Platelets to Hemostasis etc Serotonin	39
2 Thrombocytes	39
3 Platelets and Clot Retraction	40
a) Clot retraction	40
b) Role of platelets	41
4 Clotting Factors in Blood Platelets	43
a) Need of platelets for blood clotting	43
b) Erroneous ideas	43
c) Platelet 'thromboplastic' factor (prothrombin 'activator')	43
d) Platelets and the activation of prothrombin	44
e) Platelets and prothrombin consumption	45
f) Platelets and thromboplastin generation: intermediates	46
g) Platelet factors related to clotting etc	49
h) Erkelens' thesis	51

PART II EXPERIMENTAL

A	<u>GENERAL PURPOSE</u>	53
B	<u>GENERAL METHODS</u>	53
1	Blood Collection	53
a)	Dog	53
b)	Human	53
c)	Bovine	54
2	Preparation of Serum Plasma Platelets	54
3	BaSO ₄ adsorption etc	54
4	Fibrinogen	55
5	Prothrombin	55
a)	Howell type	55
b)	Seegers type	55
c)	Eluates	55
C	<u>REAGENTS</u>	55
1	Commercially Available Products	55
2	Testing of Reagents	56
a)	Fibrinogen assays	56
I)	Gravimetric	56
II)	Colorimetric	56
III)	Turbidimetric	56
b)	Prothrombin time determinations	57
c)	Thrombin forming factors assayed by <u>one stage</u> methods	57
d)	Method I specific prothrombin assay	57
e)	Specific proaccelerin assay (1 stage)	58
f)	Specific proconvertin assay (1 stage)	59
g)	Method II: orthodox <u>two-stage</u> prothrombin assay 'Improved'	59
h)	Method III BaSO ₄ citrate <u>eluate</u> technique	59
i)	For prothrombin	60
j)	For 'proconvertin index'	60
k ₁ k ₂)	For accelerator in plasma platelets etc	60
l ₁ l ₂)	For thromboplastin in tissue extracts platelets etc	61
D	<u>INHIBITOR MECHANISMS</u>	61
1	Heparin Assays	61
a)	Whole blood clotting times	61
b)	Variants of protamine titration methods	61
2	Antithrombin tests	61
a)	Plasma thrombin clotting times	61
b)	Serum antithrombin	61
E	<u>STUDIES ON ARTIFICIAL CLOTTING SYSTEMS</u>	61
1	Test Methods	61
2	Introduction: 'clotting time'	62
3	The Thrombin Fibrinogen Reaction	62
a)	Fibrinogen and clotting time	62

b) Standardization of thrombin	62
c) Standardization of fibrinogen	62
d) Thrombin concentration and clotting time	63
4 The 'Inverse Law'	63
F EXPERIMENTS	63
1 Experimental Conditions Modifying the Thrombin Fibrinogen Reaction	63
<u>Experiment 1</u> (1956) Table II Figure 13	64
Thrombin fibrinogen clotting times and 'serum factor'	
<u>Experiment 2</u> (1956) Table III	65
Calcium effects on the thrombin clotting of fibrinogens with decreasing content of 'serum factor'	
2 Experimental Evidence that Calcium is not an Essential Component of the Thrombin Fibrinogen Reaction	65
<u>Experiment(s) 3</u> (1937 1956) (cf Table XX)	65
Clotting of thrombin and fibrinogen after decalcification by (a) oxalation and electro dialysis (b) ion exchange resins	
3 Experiments on the Clotting of Platelet-Free Plasma	66
<u>Experiment 4</u> (1949) Tables IV V	66
Successful preparation of completely platelet-free plasma	
Testing of reagents which can cause it to clot	
4 Experiments with the Berkefeld (Bacteriological) Filter Technique Older experiences (cf Experiment 22)	69
5 Experiments with the Benzene Extraction Technique and its Relation to 'Available' Cephalin	70
<u>Experiment 5</u> (1934)	70
Benzene extraction to remove the coagulability of prothrombin + fibrinogen + Ca mixtures	
<u>Experiment 6</u> (1956) Table VIII with assays on <u>eluates</u> (Tables VI and VII)	70
Benzene extraction of <u>eluates</u> and fibrinogen; study of the effects on a one stage ('synthetic plasma') system	
<u>Experiment 7</u> (1956) Table IX	73
Confirming Experiment 6 and also testing for the clot promoting activity in the benzene extractives	
<u>Experiments 8 and 9</u> (1956) Tables X XI	74
Study of effects of benzene extraction on the two stage (Method III) system of thrombin formation from pro thrombin(etc) containing <u>eluates</u> ; effects of the benzene extractives	
6 Experiments on Thrombin Formation	75
(6 A) Reference Standards	75
<u>Experiment 10</u> (1956) Table XII Figure 14	75
Method III two-stage; reference assays (thrombin yields) at various dilutions of <u>eluate</u>	
(6 B) Antihemophilic Globulin in Relation to Thrombin Formation by Cephalin and by Tissue Thromboplastin	77
<u>Experiment 11</u> (1956) Table XIII	77
Effects of purified AHG (antihemophilic globulin) on thrombin formation in relation to (a) cephalin and (b) tissue thromboplastin PTC questions	

(6 B ₁) The Thromboplastin Generation Test	78
(6 C) A Calcium Containing 'Intermediate' in Thrombin Formation?	79
<u>Experiment 12</u> (1956) Table XIV	79
Decalcification at various times during thrombin formation from eluate Sequestrene Na ₂ as decalcifying agent cephalin as thromboplastin' AcG added	
<u>Experiment 13</u> (1956) Table XV	81
Confirmation of Experiment 12 on <u>dialyzed</u> eluate and with tissue thromboplastin	
<u>Experiment 14</u> (1937) Table XVI	82
Effects of oxalate 'decalcification' at various stages of thrombin formation from Howell type prothrombin with added cephalin and calcium	
<u>Experiment 15</u> (1937) Table XVII	82
Similar to Experiment 14 but using citrate	
<u>Experiment 16</u> (1947) Figure 15	83
Effects of oxalate 'decalcification' at various stages of thrombin formation from highly purified (Seegers) p-rothrombin activated by calcium and tissue thromboplastin	
<u>Experiment 17</u> (1956) Table XVIII	84
Effects of cationic exchange resin (Dowex '50') decalcification at various stages of thrombin formation from eluate activated with calcium and cephalin Attempted reactivation	
(6 D) A Lipid Containing 'Intermediate' in Thrombin Formation?	85
<u>Experiment 18</u> (1956) Tables XIX XX	85
Effects of benzene extraction at various stages of thrombin formation from eluate activated with AcG cephalin and calcium	
Summary of Data on the Postulated 'Intermediary Complex' (Experiments 12-18)	86
(6 E) Fate of Proconvertin during Thrombin Formation Preliminary Experiment (17a)	86
(6 F) Quantitative Relationships of Cephalin (Varying Concentrations) to Rate and Yield of Thrombin Formation	87
<u>Experiment 19</u> (1955) Table XXI	87
Reference series of cephalin dilutions Method III	
<u>Experiment 20</u> (1938) Table XXII	89
Effects of varying cephalin concentrations on the activation of Howell type prothrombin	
(6 G) Experiments with Tissue Thromboplastin	89
<u>Experiment 21</u> (1948) Table XXIII	89
Effects of varying tissue thromboplastin concentrations on thrombin formation from purified (Seegers') prothrombin	
<u>Additional Experiments</u> with Tissue Thromboplastin	90
(6 H) Some Modern Assays and Tests on Howell Type 'Prothrombin' with Reference to Factors Which May Affect Thrombin Formation	90

<u>Experiment 22</u> (1956) Tables XXIV A XXIV B with 1 stage assay reference curves (Figure 16) for (a) pro- thrombin times (b) prothrombin (c) proconvertin (d) proaccelerin in dog plasma Assays for (1) fibrinogen (2) thrombin (3) pro thrombin (4) proconvertin (5) proaccelerin (6) antithrombin (7) AHF (8) PTC Comparison with precursor plasmas	91
(6 I) Quantitative Relationships of Calcium in Thrombin Formation	92
<u>Experiment 23</u> (1956) Table XXV Effects of decreasing Ca on activation of eluate pro thrombin (+ proconvertin) in presence of AcG and tissue thromboplastin	92
<u>Experiment 24</u> (1956) Table XXVI Effects of increasing Ca on activation of same system as in Experiment 23	93
<u>Experiment 25</u> (1948) Table XXVII Effects of varying Ca on activation of Seegers' prothrombin (rich in proconvertin but poor in AcG) in presence of tissue thromboplastin	94
(6 J) The Question of 'Spontaneous' Activation of Prothrombin to Thrombin Trace Contaminants? Stability of Pro thrombin and Thrombin	95
<u>Experiment 26</u> (1948) Table XXVIII 'Spontaneous' thrombin formation in highly purified (Seegers') prothrombin	95
<u>Experiment 27</u> (1948) Table XXIX Study of stability of Seegers' prothrombin in solution	96
<u>Experiment 28</u> (1948) Table XXX Further study of stability of Seegers' prothrombin in solution in presence of Ca ions and thrombin	97
(6 K) Testing for (A) Thromboplastic and (B) Antithrombo- plastic Activity in Various Lipoidal Materials Par- ticularly Cephalin and Synthetic Phosphatides and Their Contaminants	97
<u>Earlier Data</u> Table XXXI	97
<u>Current (1956) Data</u> Table XXXII	98
(6 L) Actions of Heparin and Heparin Cofactor with Special Reference to the Thrombin Forming Reactions (Anti- thromboplastic)	100
<u>Experiment 29</u> (1939) Table XXXIII Effects of heparin on activation of Howell type prothrombin in presence of Ca and (1) cephalin (2) rabbit brain thrombo- plastin (3) brain F lipids; (4) crystalline trypsin	100
<u>Experiment 30</u> (1941) Table XXXIV Significance of heparin cofactor in inhibition of thrombin formation	101
<u>Experiment 31</u> (1948) Table XXXV Demonstration of 'antithromboplastic' action of heparin in delaying thrombin formation from purified (Seegers') prothrombin	103
Considerations Regarding Modes of Action of Heparin	103

(6 M) Thromboplastic Enzymes in Relation to Thrombin	
Formation by Thromboplastic Lipids and (7) Lipoproteins	104
Foreword 1939 Data	104
<u>Experiment 32</u> (1948) Figure 17	105
Demonstration of need for calcium and thromboplastin in the	
trypsin activation of purified (Seegers') prothrombin	
<u>Experiment 33</u> (1948) Table XXXVI	106
Further studies on thrombin yields in the presence of trypsin	
(6 N) Plasmatic Thromboplastin Components Concerned with	
Thrombin Formation PTC and AHF	107
<u>Experiment 34</u> (1956) Table XXXVII	107
Tests for PTC and AHF on (I) dialyzed eluate (II) Howell	
type prothrombin and (III) purified AHG	
7 Testing of Clotting Factors in Blood Platelets	108
(7 A) Thromboplastic Action of Platelets Potentiation by	
(a) AHG (b) Trypsin (c) Fibrinolysin and (d) Weak Thrombin	108
<u>Experiment 35</u> (1936) Table XXXVIII	108
Thrombin formation from eluate in presence of platelets	
Thromboplastic potentiation by (a) AHG; (b) trypsin	
<u>Experiment 36</u> (1956) Table XXXIX	109
Potentiation of platelet thromboplastin by (c) fibrin	
olysin and (d) weak thrombin	
<u>Older Experiments</u> (1936 etc.)	110
Reagents 1947 1949	111
<u>Experiment 37</u> (1949) Table XL	112
Thrombin formation from Seegers' prothrombin in presence	
of Ca AcG and platelets Potentiating effects of (a) thrombin;	
(b) fibrinolysin	
(7 B) Effects of Anti Proteases on Various 'Thromboplastic'	
Additives in Thrombin Forming Systems	113
Preliminary Studies	113
Facts needed for interpretation of experiments	
<u>Experiment 38</u> (1948) Table XLI	114
To test anti proteases (P I Afln) for effects on thrombo	
plastic action of platelets + trypsin in thrombin forming	
systems	
<u>Experiment 39</u> (1948) Table XLII	114
To test anti proteases (P I Afln S B I) for effects on	
thromboplastic action of platelets + fibrinolysin	
<u>Experiment 40</u> (1956) Table XLIII	115
To test anti proteases (S B I P I N B I Afln) in	
thrombin forming systems in which the thromboplastic	
additives are (I) platelets; (II) platelets + AHG	
<u>Experiment 41</u> (1948) Table XLIV	116
To test anti proteases (S B I P I Afln) in thrombin	
forming systems activated by Ca + tissue thromboplastin	
<u>Experiment 42</u> (1956) Table XLV	116
To test anti proteases (S B I P I N B I) in thrombin	
forming systems activated by Ca + cephalin	
General Discussion of Experiments 38 42	116

<u>Experiment 22</u> (1956) Tables XXIV-A XXIV B with 1 stage assay reference curves (Figure 16) for (a) pro thrombin times (b) prothrombin (c) proconvertin (d) proaccelerin in dog plasma Assays for (1) fibrinogen (2) thrombin (3) pro thrombin (4) proconvertin (5) proaccelerin (6) antithrombin (7) AHF (8) PTC Comparison with precursor plasmas	91
(6 I) Quantitative Relationships of Calcium in Thrombin Formation	92
<u>Experiment 23</u> (1956) Table XXV Effects of decreasing Ca on activation of eluate pro- thrombin (+ proconvertin) in presence of AcG and tissue thromboplastin	92
<u>Experiment 24</u> (1956) Table XXVI Effects of increasing Ca on activation of same system as in Experiment 23	93
<u>Experiment 25</u> (1948) Table XXVII Effects of varying Ca on activation of Seegers' prothrombin (rich in proconvertin but poor in AcG) in presence of tissue thromboplastin	94
(6 J) The Question of 'Spontaneous' Activation of Prothrombin to Thrombin Trace Contaminants? Stability of Pro thrombin and Thrombin	94
<u>Experiment 26</u> (1948) Table XXVIII 'Spontaneous' thrombin formation in highly purified (Seegers') prothrombin	95
<u>Experiment 27</u> (1948) Table XXIX Study of stability of Seegers' prothrombin in solution	95
<u>Experiment 28</u> (1948) Table XXX Further study of stability of Seegers' prothrombin in solution in presence of Ca ions and thrombin	97
(6 K) Testing for (A) Thromboplastic and (B) Antithrombo- plastic Activity in Various Lipoidal Materials Par- ticularly Cephalin and Synthetic Phosphatides and Their Contaminants	97
<u>Earlier Data</u> Table XXXI	97
<u>Current (1956) Data</u> Table XXXII	98
(6 L) Actions of Heparin and Heparin Cofactor with Special Reference to the Thrombin Forming Reactions (Anti- thromboplastic)	100
<u>Experiment 29</u> (1939) Table XXXIII Effects of heparin on activation of Howell type prothrombin in presence of Ca and (1) cephalin; (2) rabbit brain thrombo- plastin (3) brain F lipids; (4) crystalline trypsin	100
<u>Experiment 30</u> (1941) Table XXXIV Significance of heparin cofactor in inhibition of thrombin formation	101
<u>Experiment 31</u> (1948) Table XXXV Demonstration of 'antithromboplastic' action of heparin in delaying thrombin formation from purified (Seegers') prothrombin	103
Con siderations Regarding Modes of Action of Heparin	103

G CLINICAL CASES WITH PLATELET PROBLEMS	130
Foreword	130
Cases:	
Group I Table LI	
(A) Normal adults (>50 cases)	131
(B) Parturient mothers (32 cases)	132
(C) Normal newborn infants (cord bloods) (32 cases)	132
Group II Table LIII-A	
Congenital (familial) thrombocytopenia (Cases 1-2)	132
Group III Table LIII-B	
Hereditary thrombocytopathic purpuras 3 cases viz	
Glanzmann's Thrombasthenia (Case 3)	133
Pseudohemophilia with Platelet Problems (Cases 4-5)	133
Acquired Deficiencies of Platelet Functions:	
Group IV Table LIV	
Acquired Thrombocytopenia 25 cases viz	
Cases 6-16 acute idiopathic thrombocytopenic purpura	134
Cases 17-23 chronic idiopathic thrombocytopenic purpura	134
Cases 24-28 associated with aplastic anemia (2 cases)	
macrocytic anemias (2 cases) Banti's	
syndrome (1 case)	134
Cases 29-30 associated with metastatic bone cancer	
(primary in prostate)	134
Group V Table LV	
Thrombocytosis 3 cases viz	
Case 31 associated with generalized carcinoma	
(primary in stomach)	135
Case 32 associated with polycythemia rubra vera (and gout)	135
Case 33 associated with leukemia	135
Group VI Tables LVI-A LVI-B	
Leukemias with Thrombocytopenia or Thrombocytopathia	
34 cases (out of 39 examined)	
(A) Cases 34-56 thrombocytopenics	135
(B) Cases 57-67 thrombocytopathics	136
Group VII Tables LVII-A LVII-B	
Uremia with platelet problems 11 cases (out of 12 examined)	
(A) Cases 68-77 with normal platelet counts but evidence	
of functional defects	136
(B) Case 78 with 'borderline' platelet accelerator	
(chronic pyelonephritis in polycystic kidneys)	136
Group VIII Table LVIII	
Platelet problems in rare cases with primary plasma clotting	
difficulties 7 cases in a total of 52 hemophilics 26 PTC	
deficients 24 acquired hypoprothrombinemics 5 congenital	
hypoproconvertinemics 4 congenital hypoproaccelerinemics	
and 8 cases with circulating anti AHF or anti PTC etc	
Cases 79-83 with severe liver disorders 3 showing	
fibrinolysin	137
Case 84 with severe congenital hypoproaccelerinemia	
showing deficient platelet accelerator	137
Case 85 with circulating fibrinolysin associated with	
prostatic carcinoma and metastases	138

(7 C) Control Testing of Enzymes and Anti Proteases	117
<u>Experiment 43</u> (1948) Table XLVI	117
To show that anti proteases (P I S B I N B I Afln)	
have no effect on the thrombin fibrinogen reaction	
<u>Experiment 44</u> (1956) Table XLVII(A)	117
Proteolytic (fibrinogenolytic) activity of an old trypsin	
preparation and its inhibition by anti proteases (N B I P I)	
<u>Experiment 45</u> (1956) Table XLVII(B)	118
Proteolytic (fibrinogenolytic) activity of an old fibrino	
lysin preparation and its inhibition by anti proteases	
(N B I and Afln)	
(7 D) Fibrinolytic I phenomena and Platelets	119
Platelet anti protease inhibitory to (a) trypsin and	
(b) fibrinolysin	
(7 E) The 'AcG Like' Factor in Platelets	119
<u>Experiment 46</u> (1950) Table XLVIII	119
Comparison of 'AcG like' potency of washed platelets with	
a serum AcG preparation (Ware & Seegers)	
(7 F) Effects of Heparin on Thrombin Formation by (I) Platelets;	
(II) Platelets + Trypsin; (III) Tissue Thromboplastin	120
<u>Experiment 47</u> (1956) Table XLIX	120
Heparin inhibition of the thromboplastic action of (I) platelets;	
(II) platelets + trypsin; (III) tissue thromboplastin	
(7 G) Analytical Fractionation of Platelets	121
Lipid analyses	
(7 H) Question of a 'Fibrinoplastic' Factor in Platelets	121
<u>Experiment 48</u> (1956) Table L	122
Absence of effects of platelets on the thrombin fibrinogen	
reaction	
(7 I) Platelet Utilization during Clotting	122
<u>Experiment 49</u> (1955) Table LI	122
Rate of disappearance of platelets during clotting of	
(A) normal and (B) hemophilic blood	
8 Dark Field Microscopic Observations of Phospholipids	
Platelets and Other Formed Elements of the Blood and	
Bone Marrow	123
(8 A) Foreword Earlier work	123
(8 B) Platelets	123
1) Alteration during blood coagulation Figure 18	123
2) Agglutination of platelets	125
3) Platelets and fibrin	126
4, Platelet preservation	126
a) Anticoagulants	126
b) Recalcification and role of calcium in	
platelet alterations	127
(8 C) Megakaryocytes Figure 19 (plates 5 6)	127
(8 D) Thrombocytes Figure 19 (plates 7 8)	128
(8 E) Erythrocyte Stromatolysis etc Figure 20 (plates 9 10 11)	128
(8 F) Myelin Figure Formations from Phospholipids	
Figure 20 (plates 12A 12B)	129
(8 G) Conclusion	130

THROMBOPLASTIN FACTORS: <u>Experiment 55</u>	Figure 26	156
<u>Experiment 56</u>	Table LXI	156
<u>Experiment 57</u>	Figure 27	156
STYPVEN <u>Experiment 58</u>	Table LXII	157
<u>Experiment 59</u>	Tables LXIII LXIV	158
DISCUSSION		159
<u>Experiment 60</u>	Figure 28	159
<u>Experiment 61</u>	Table LXV	159
TRYPsin and THROMBIN		159
FINAL INTEGRATION OF IDEAS		160
APPENDIX I Figures and Illustrations (1-27): Captions		163
APPENDIX II Tables (I LXV)		195
APPENDIX III Bibliography (pp 249-278)		249

Group IX Table LIX

Platelet problems in radiation sickness

Case 86 cancer case under treatment with Au¹⁹⁸ 138

Experiment 50 (1952) Table LX

139

Platelet studies and clotting tests (especially pro
thrombin consumption) on 5 dogs (and 2 controls)

Au¹⁹⁸ in doses of 1 & 20 millicuries/kg

PART III INTEGRATION OF IDEAS

A FORMULATION

141

1 Thromboplastic Lipid

141

2 Natural Inhibitors

141

3 Cephalin

141

4 Lipid Antithromboplastin

142

5 'Completeness' of Thromboplastin

142

A new way to demonstrate 'thromboplastin generation'

6 Intermediates

143

7 The Cephalin 'Availability' Theory

143

8 Thromboplastic Enzymes

144

9 The Fibrinolysin Question

144

10 Platelets

144

11 The Trigger Mechanism

145

12 Clinical Disorders of Platelets

145

13 Myelin Forms

146

14 Preservation of Platelet Thromboplastic Component

146

15 Potentiation of Platelet Thromboplastin

146

B FINAL SUMMARIZATION

147

PART IV SUPPLEMENT

149

THE VERSATILE TWO-STAGE METHOD FOR QUANTITATIVE
STUDY OF BLOOD CLOTTING FACTORS

149

TECHNIQUE FOR THE VERSATILE TWO-STAGE

149

SPECIFIC FACTOR ASSAYS

151

PROTHROMBIN Experiment 51 Figures 21 22

151

ACTIVATORS

152

FACTOR VII Experiment 52 Figure 23

153

HAGEMAN FACTOR Experiment 53 Figure 24

154

STUART FACTOR Experiment 54 Figure 25

155

OTHER ACCESSORY FACTORS

155

LIPOIDS AND BLOOD PLATELETS

WITH REFERENCE TO BLOOD COAGULATION

AND THE HEMORRHAGIC DISEASES

PART I

BACKGROUND

FOREWORD

In presenting a dissertation embodying experimental work conducted over a 27 year period (1929-1956) it is necessary to review the knowledge of the field at the commencement of this period and its subsequent progress to the present time in order to evaluate each contribution in the light of contemporary knowledge and theory. Several volumes and possibly several authors would be required to review critically and in detail all the extensive literature of the blood coagulation field. From the physiological approach alone several thousand titles are referred to in the monumental reviews of P Morawitz [346-347] and E Wohlsch [505-506] and in more recent articles including those appearing in the Annual Reviews of Physiology two of them by the present author [133-140]. The purposes of this dissertation may best be served by a selection of those ideas and trends of thought which afford the background for the topics presented.

A dissertation more than a definitive scientific publication reflects the interplay of ideas that influence the mind of its author and it is not inappropriate that it indicate to some extent the personal element in research. In the period covered Science itself has undergone a change which is deserving of mention. This change was gradual in the beginning but it has accelerated in the last few decades of the twentieth century. Characteristic of earlier periods was the eminence of individual scientists whose discoveries opened up new fields or special areas within those fields often single handed. Students and young investigators came to work in the laboratories of the distinguished authority and thus developed the various 'schools' justly adding to the reputation and prestige of the 'master'. A fine example in our field was the Dorpat school under the leadership of Alexander Schmidt (1860-1895).

The modern scientific era has seen much larger numbers of scientists working simultaneously in the same field. Through scientific gatherings of regional, national and international scope workers of kindred interest have frequent opportunities for exchange of ideas. Thus all are kept fully informed of many lines of progress and of the definition of numerous problems in advance of their solution. A not infrequent consequence is that the same solution (or almost) is reached simultaneously by several independent groups of workers perhaps widely scattered over the world. An illustration of this may be cited from a field peripheral to that of the present inquiry. In 1939 the Journal of the American Chemical Society simultaneously published four papers from different laboratories all of which must share the credit for the definitive solution to the problem of the chemistry of vitamin(s) K. Much the same sort of thing is true of the recent

mention. These established the clotting phenomenon as essentially a physiological property of the 'coagulable lymph' (Butt 1760 cf. 'coagulated lymph' of de Senac 1749) which we nowadays call plasma. Malpighi (1666) and Ruysch (1707) had already noted the 'fibrous material obtainable from blood clots' and clinicians with wide experience of ubiquitous blood letting talked of the 'cruur' or crassamentum appearing after separation of the 'serosity' or serum. Much was made of the 'buffing' of blood in disease. This we now interpret as an increased erythrocyte sedimentation rate with the consequent easier observation of clotting sequences starting in the supernatant plasma rich layer. The term fibrin which we owe to the French encyclopedists Chaptal [72] and de Fourcroy [99] was not really an advance since they used the crudest methods of preparation of the clot material which they tried to characterize by appearance and elementary analysis. Mulder introduced the word protein in 1839 but it has been a long and difficult road still with unexplored bypaths to define individual proteins and among them fibrin and fibrinogen. The latter term we owe to Virchow [482].

The 1830 1859 publications of P. S. Denis are of historical interest. Denis' [102] most significant contribution all things considered was the practical preparation of his 'plasmine'. This was a precipitate obtained from plasma with strong solutions of sodium chloride and other neutral salts. Redissolved on addition of water it subsequently underwent coagulation indistinguishable from the appearance of natural fibrin clot.

The false impression that fibrin was the result of some 'spontaneous' change in a soluble precursor (fibrinogen) was first questioned by Buchanan [68 69] particularly in his observation that the washings from a blood clot could coagulate certain hydrocele fluids. He compared this with the rennet curdling of milk and also did much to try and prove a cellular origin of the blood coagulant. Buchanan's work was neglected until republished by Gamgee [183] in support of his confirmatory experiments. Gamgee's method of preparing thrombin (as it has come to be termed) from blood clots was further improved by W. H. Howell [229 232] at the beginning of our current era.

2 THE DORPAT SCHOOL

Alexander Schmidt of Dorpat established his famous school in the latter half of the 19th century. From the master and his pupils came a large body of work and publications reviewed in Schmidt's two monographs [420 421] in 1892 1895. It is easy from our advanced modern viewpoint to be extremely critical of the crudity of their experimentation and highly theoretical presentations of their ideas. It should be remembered however that the difficult biochemical problems of the coagulation field humble us even to this day. Then again the 19th century discoveries especially in the field of ferments (or enzymes) aroused such interest that contemporary enthusiasm sometimes got out of bounds. Analogies were suggested and their implications tacitly accepted with little of the caution demanded of more recent experimental science. Schmidt used a crude technique of storing blood serum under alcohol to obtain his 'fibrin ferment' which was a very weak

discoveries of several new factors in the blood clotting mechanism. Here another consequence is manifest namely some uncertainty because of the difficulty in exact identification of the factors in question thus leading to a confusion in nomenclature. Such confusion is more troublesome to the casually interested scientist than to those actually working on these problems however.

In the coagulation field at least in America perhaps the last of the old school authorities was W. H. Howell of the Johns Hopkins School of Medicine in Baltimore. This eminent scientist and distinguished teacher was quietly continuing his critical experimentation in a modest laboratory which he occupied as Emeritus Professor of Physiology during the period of the 1930's when the present author had the privilege of paying him numerous visits. His friendly charm, advice and critical discussions and warm personal interest and encouragement were of inestimable value in guiding a new researcher into the complexities of the blood clotting field.

On the occasion of the annual meeting of the Federation of American Societies for Experimental Biology at Detroit in 1935 a small group of interested coagulationists met together and agreed to maintain an exchange of ideas. By common consensus this 'coagulationist' group has never become formally organized. It has however continued to meet at each annual meeting of the Federation and the sustained interest has expanded with each succeeding year so that our mailing list now has over two hundred names. There are similar groups of workers in other specialized fields and a very important result of their coordinated interests has been to secure inter society sessions and symposia at such gatherings as the Federation and the International Physiological and Hematological Societies' meetings. Other significant contributions to the cross fertilization of ideas among specialized scientific groups are the numerous Research Conferences which are a distinctive feature of the modern scientific scene. Some examples in which American coagulationists get together are those promoted by the National Research Council, the U. S. Public Health Service and the Josiah Macy Jr. Foundation to mention just a few.

With this broad perspective we may selectively summarize the early history of blood coagulation in what we may call the personal era and then proceed to a brief review of its modern status in which due credit for individual investigators need not be denied by presenting this largely from the depersonalized point of view.

The topic of the blood platelets will be separately reviewed.

CHANGING CONCEPTS IN THE CLOTTING FIELD

A HISTORICAL HIGHLIGHTS

1. EARLY ORIGINS; HEWSON, DENIS BUCHANAN

The earliest significant efforts toward an understanding of the coagulation of the blood were made in the 18th century and the Experimental Inquiries of young William Hewson [218], associate of the famous brothers John and William Hunter, are particularly worthy of

3 THE WORK OF HAMMARSTEN AND WOOLDRIDGE

Olof Hammarsten [205] in 1883 seemed to simplify Schmidt's theories by suggesting that 'fibrinoplastic substance' ('fibrinoglobulin') was not a component of the coagulant but appeared as a split product of fibrinogen when acted upon by the fibrin ferment. However this eminent biochemist went on to considerable success in the purification of both fibrinogen and thrombin. We owe to him the application of the 'salting out' technique to precipitate fibrinogen from plasma using $\frac{1}{2}$ sat NaCl or $\frac{1}{4}$ sat $(\text{NH}_4)_2\text{SO}_4$. Weyl Heynslus and others (ref [371]) were then developing the use of neutral salts rather than the earlier acids and alkalis etc for the separation of all kinds of proteins. The distinction between albumins and globulins on the basis of solubilities in such salt solutions was made in this era. When oxalate or citrate (see later) replaced the older MgSO_4 etc as anticoagulants used in separating (centrifugally) the plasma from the blood corpuscles the preparation of fibrinogen and other clotting factors by the salting out method was much improved. The technique of elementary organic analysis by combustion methods dating back to Liebig (1840) was continued and despite the relative crudity of the materials analyzed did give data on N S P and minerals e.g. Ca which contributed some information of real value in these early days of protein and other organic chemistry. Hammarsten eventually purified fibrinogen until he was able to obtain up to 94 percent of its protein in the fibrin clot which resulted from treating its solutions with thrombin. His thrombin was fractionated from fresh horse serum. The conclusion from these careful experiments was that the small quantity of non coagulable protein unaccounted for was merely a minor contamination with what we would now term serum globulins. Hammarsten's work on the calcium factor will be reviewed on p. 8.

L. C. Wooldridge [508] had considerable justification for some of his claims to priority over the Dorpat school in the investigation of phospholipids ('lecithins') in the blood clotting system. He obtained many interesting experimental facts but his tissue fibrinogen theory and many aspects of his work were too unorthodox to gain him wide recognition. Even the publication of his obscure works by Horsely and Starling in 1893 failed to shake the widespread acceptance of the thrombin theory.

4 THE CALCIUM FACTOR Early work of Arthus Pekelharing Hammarsten Sabbatini

Alexander Schmidt was never convinced of the need for calcium in the thrombin forming reaction. There had been previous suggestions [201-404] for a role of calcium in blood clotting but the significant evidence was provided by Arthus and Pages in 1890 [19]. They used oxalates or fluorides to prevent clotting and restored it by simple re-addition of calcium salt.

Pekelharing [372-373] confirmed this and introduced citrates for the same purpose. This worker improved upon Schmidt's methods for obtaining prothrombin. Dilution and acidification of plasma with weak

and impure thrombin by modern standards. His numerous experiments endeavored to search out all possible factors influencing the clotting mechanism. 'Proplastic' i.e. fibrinogen containing liquids such as hydrocele fluid or plasma obtained from blood kept fluid with $MgSO_4$ and other neutral salts (cf. Hewson [218]) were clotted by Schmidt's fibrin ferment. He obtained good evidence for a precursor of the coagulant. The terms thrombin (for fibrin ferment) and prothrombin (for its precursor) were introduced in his later writings. Thus Schmidt has deservedly been called the father of the thrombin theory. Many details of his particular theories and terminologies have long since been discarded and need not be reviewed. A few words may be said however about some of the experimental facts. In attempting to unravel the thrombin problem, Schmidt did much to implicate (he thought) both (1) a plasma protein 'paraglobulin' and (2) tissue or cellular factors which he called 'zymoplastic substances'. As to the first, it did seem rational at the time to look for a proteolytic 'split product' accompanying the postulated enzymatic breakdown of fibrinogen to fibrin.

Schmidt and his pupils did a great deal with the tissue materials noted under the second head, namely 'zymoplastic substances'. These were obtained both in aqueous extracts and by means of fat solvents, hence chiefly lipoidal in character. Heat stability and many other properties were investigated. The most significant conclusion about these zymoplastic agents was that they were not thrombin but could participate in the activation of prothrombin.

The essential technique whereby Schmidt sought to characterize tissue fractions was first to obtain an alcoholic extract ('zymoplastic substance'), then a succeeding watery extract ('cytoglobulin') and its acetic acid precipitable fraction ('preglobulin') and finally an insoluble residue ('cytin'). Of historical interest were the observations (1) zymoplastic substance promoted clotting under a variety of test circumstances, some of which led to the conclusion (above) that it could participate in the activation of prothrombin to thrombin; (2) the defatted cellular extractives, especially preglobulin, had considerable clot inhibiting powers, e.g. for mixtures of $MgSO_4$, plasma and fibrin ferment (thrombin); (3) zymoplastic substance however was able to restore clotting in these inhibited mixtures.

Schmidt's final summing up of his views on coagulation [421] included the idea of something normally present in blood which could be regarded as an antithrombin. This term was actually introduced by Fano (1881) to designate the thermolabile (on boiling) agent which caused incoagulability of blood after peptone injections in the dog, as first shown in Carl Ludwig's laboratory by Schmidt Mulheim (1880). Extracts from the medicinal leech contain a substance hirudin which Haykraft [217] showed to have similar 'antithrombic' powers.

It was generally known in this era that (a) the 'thrombic' action of fresh serum progressively decreased on aging even for a few hours; (b) thrombin clotted plasma less readily than a fibrinogen solution; (c) the weak thrombin preparations of that time had little if any capacity to produce coagulation (thrombosis) on intravenous injection into experimental animals.

Even when the blood remained coagulable the clots would soon undergo lysis. As little as 1/60 vol of normal dog plasma could prevent this indicating that normal plasma contains a powerful antifibrinolytic. This however is reduced or absent in the above cases involving failure of liver function. Nolf performed many noteworthy experiments but his theories were heterodox. In brief he tried to bring into a dual clotting fibrinolytic scheme: (1) fibrinogen (2) 'thrombogen' which is possibly equivalent to the orthodox prothrombin although Nolf also identified it with the antifibrinolytic agent (3) 'thrombozyme' possibly equivalent to Schmidt's xymoplastic substance or Morawitz's thrombo kinase since Nolf asserted that it was produced by certain white cells (thrombocytes and platelets) and by the endothelium of the capillaries. Nolf accepted thrombin only as a by product of the reaction between the above three substances and not as the coagulant itself as in the thrombin theory. He used the term 'thromboplastic' for a heterogeneous group of agents including wettable surfaces e.g. ground glass, calcium oxalate in colloidal suspension, chloroform and similar emulsification which assist in the colloidal reactions. The thromboplastic agent intervenes solely to make the reaction possible between these [3 cited] precursors. It acts as a catalyst. And so also do the alcoholic or aqueous tissue extracts. It may be remembered that Freund [174] and Bordet & Gengou [58] retarded blood and plasma clotting by use of paraffined surfaces.

7 THE HOWELL THEORY

Around 1910 W. H. Howell and his pupils were re-investigating (a) the thrombin-fibrinogen reaction (Rettger 1910; Howell 1910), (b) the intravenous injection of thrombin (Davis 1910) and (c) the clotting of peptone and hirudin plasmas especially by glycerinated (Cecil 1911) tissue extracts. In a 1911 paper Howell [230] carefully reconsidered the role of thromboplastic tissue extracts and because of their ability to clot the above mentioned inhibitor-containing plasmas he concluded in favor of an alternative to Morawitz's theory namely that tissue extracts act only by neutralizing certain clot inhibitors. Furthermore he argued for a type of (natural) inhibitor which does not merely antagonize active thrombin but actually prevents the formation of thrombin from prothrombin. Hence the new term 'antiprothrombin' and the specific idea (Howell's 'thromboplastin') that tissue extracts particularly the thermostable lipoidal fraction act by neutralizing antiprothrombin.

In 1912 independent of Zak [514] Howell [231] identified the thromboplastic agent as cephalin in some form of protein combination. Lecithin whether from tissues or egg yolk was inactive. Howell's cephalin preparation followed the method of Thudichum [464-465] pioneer in the field of brain chemistry. Diakonow had separated 'lecithin' from other brain lipid fractions and shown that its hydrolytic products were glycerophosphoric acid choline and fatty acids such as oleic and margaric (ref. [103]). Thudichum found that there were a variety of phosphorylated lipids (phosphatides; phospholipids) differing especially in their nitrogenous basic groups. Retaining the name lecithin for those containing choline the new term cephalin was given to

acetic acid separated this agent by a principle which we would now characterize as isoelectric precipitation. Pikelharing however went to undue lengths to identify his material as a 'nucleo albumin' and suggested that it had a cellular perhaps platelet origin. These were not useful ideas and they somewhat lessen the value of his contributions. He did insist upon the importance of calcium salts in activating prothrombin to thrombin (fibrin ferment) but he loses us in his further speculations about its transfer of calcium to the fibrinogen in the act of clotting.

Hammarsten [206] provided more convincing evidence of the specific importance of calcium in prothrombin activation while denying it any really significant role in fibrin formation. By oxalation and 'isoelectric' precipitation of his fibrinogen and thrombin he obtained reagents which interacted to give a fibrin analyzing as little as 0.005 percent calcium. This could reasonably be dismissed as a trace impurity (but see Howell's comments [238]).

The question raised by citrates which are unlike oxalates in that they do not precipitate calcium was settled at the turn of the century by Sabbatini [417] who showed that they do remove Ca^{++} ions by forming inactive CaCit^- anion complexes.

5 THE MORAWITZ-FULD THEORY

Morawitz [345-346] and Fuld & Spiro [181] clarified ideas concerning the role of tissue factors in the clotting mechanism. Fuld's term 'cytozyme' was later adopted by a minority of workers notably including Bordet [55] whereas most European investigators accepted Morawitz's term 'thrombokinasin'. The argument was still by analogy with a tacitly accepted enzyme hypothesis and it may be remembered that Pavlov (1899) had just discovered enterokinase to explain the intestinal activation of pancreatic trypsinogen into active trypsin. The Morawitz-Fuld theory did clearly state that both calcium ions and tissue factor were needed to convert prothrombin into thrombin.

Morawitz [345] also gave us the term 'metathrombin' for the inactive form of thrombin in serum believed due to some combination with antithrombin which Morawitz following earlier leads by Alexander Schmidt thought he was able partly to reverse with an acid and alkali treatment.

6 THE HETERODOXY OF NOLF

P. Nolf [351] in the later 1900's restudied the phenomena of peptone shock in experimental animals particularly in relation to the liver. He not only confirmed earlier French workers (Contjean, Gley, Hedon and Delezenne) as to the hepatic origin of antithrombin (which Nolf preferred to call 'antithrombosin') but also gave good evidence that the liver produced fibrinogen and antifibrinolysin. Nolf was especially interested in the fibrinolytic phenomenon pointing out the loss of fibrinogen due to appearance of an active proteolytic enzyme (fibrinolysin) in the blood partly explaining the incoagulability encountered not only in peptone shock but also in liver poisoning with phosphorus (Corin & Anciaux 1894) or with chloroform (Doyon 1905).

of prothrombin calcium and kinase (tissue extract) Howell's opposite results were thought to be due possibly to differences in the two prothrombin preparations or in the test substrate (purified fibrinogen vs dialyzed oxalated plasma). Howell failed to mention a much more significant point namely that Mellanby's 'kinase' was a crude aqueous tissue extract whereas Howell's 'thromboplastin' was purified cephalin. Howell cites Fuchs' [177] suggestion that the prothrombin complex might be the same as the 'proserozyme' postulated by Bordet and Delange [56]. No further comment is made in the review but I know from personal conversations with Howell that he was unimpressed with this idea. Bordet's proserozyme was said to change to serozyme (prothrombin) on contact with wettable surfaces but heparinized plasma or prothrombin solutions are unaffected by the surface of the container. Howell [238] did not bring the heparin cofactor into the antiprothrombic thesis but only into the antithrombic action of heparin. Thus unlike hirudin heparin has no inhibitory effect upon the reaction between thrombin and fibrinogen although heparin added to plasma or serum causes the production in these liquids of a true antithrombin by a reaction with some unknown thermolabile constituent of the blood. Let us anticipate some of the modern era of our theme to record the reasons for the refutation of the Howell theory. This came about because of failure experimentally to verify the essential facts which the theory requires. Here are the answers to some key questions:

1) Are significant amounts of heparin to be found in the blood normally? The best answer is that of Jaques [249] who used a reliable chemical assay method which failed to find more than 10 micrograms per 100 ml in normal human bloods. This is only about 1/10 the amount of the heparin needed to keep blood from clotting in vitro. Heparinemia can occur in peptone shock and other anaphylactoid reactions [255].

2) Can heparin form a prothrombin antithrombin type of complex? Because of its highly acidic groups heparin can combine with the basic groups of many proteins etc (Flacher [162]). Chargaff [75] obtained evidence of a stoichiometric combination between heparin and protamine (a basic split product of salmon roe protein). It can form a polyanion complex with fibrinogen experimentally [42]. No one however has isolated anything like the postulated heparin prothrombin compound. The inhibitory actions of heparin have proved multiple and complex. They are still insufficiently understood but much that is now known can explain these actions without need for postulating Howell's complex [425].

3) Does thromboplastin neutralize heparin and release prothrombin? As to the first part of this question let us for the moment identify thromboplastin with cephalin (thromboplastic phospholipid). Phosphorylated lipids are acidic in much the same way as the sulfonated heparins. Hence a direct neutralization seems improbable chemically. There is much more reason and evidence (e.g. Chargaff [75]) to think of both the phosphatide and the heparin as competing for the basic groups of proteins. Cephalin combines stoichiometrically with protamine [74]. Heparin the stronger acid can 'deviate' cephalin from this combination. When heparin is made to react with the thromboplastic

those containing ethanalamine. In 1915 Howell set his pupil Jay McLean to test in the blood clotting system all phosphatides described in the literature to that time. An unexpected result will receive due consideration in a later paragraph. The data for Howell's last paper [239] on 'The isolation of thromboplastin from lung tissue' were completed just before his death in 1945 but it was his daughter Dr Janet Howell Clark who wrote it up for publication kindly submitting it for my appraisal before sending it to the publishers. This paper showed the progress Howell had made toward characterizing thromboplastin as a protein compound of a phospholipid from which it was possible to remove the protein leaving a residue with marked thromboplastic activity the chemical nature of which has not been determined.

Howell [233] used a crude preparation of prothrombin simply obtained by precipitation of plasma with acetone washing with ether drying on a Buchner funnel and subsequently extracting with alkalized water or saline. None of his prothrombins failed to activate with calcium alone. In fact many of them activated to thrombin 'spontaneously' and this was enhanced by treatment with CHCl_3 and other lipid solvents [71]. Howell [229-232] also prepared a thrombin by 8% NaCl extraction of washed blood clots (see p. 5) and could purify this by shaking with chloroform until it no longer gave any reaction to chemical tests for phosphorus. These data caused Howell to reject the European thrombokinasin idea and insist that calcium alone could activate prothrombin. Reviewing these matters in his 1916 Harvey Lecture Howell [235] agreed that thermolabile (50° – 60°C) aqueous tissue extracts or relatively thermostable cephalin do assist in prothrombin activation but he proposed an alternative explanation namely that under normal conditions the prothrombin is protected from the activating influence of the calcium ions by a combination of some kind with an inhibitory agent or antistubstance and that cephalin exerts its accelerating effect upon coagulation by neutralizing the influence of this inhibitory substance thus liberating the prothrombin so that calcium can convert it to thrombin. This is the essential Howell theory.

Howell's pupil McLean [326] in the above mentioned study of a variety of relatively crude phosphatides found some that instead of aiding had a marked inhibitory effect upon coagulation. These included two preparations of 'cuorin' (method of Erlandsen 1907) and a 'heparphosphatide' (method of Baskoff 1908). In personal conversations with Dr Jay McLean during visits to Columbus Ohio around 1940 he told me how elated he had been to discover antiprothrombin. But he had to leave for active service in World War I and missed the follow-up work in which Howell and Holt [242] and Howell [237] established the more important aspects of this anticoagulant action including need for a plasma co-factor and identified the agent not as phosphatide but as a sulfonated polysaccharide amine which Howell called heparin. In his 1925 Pasteur Lecture [236] and 1935 Physiological Review article [238] Howell clearly identified his antiprothrombin with heparin. He did note that Mellanby [330] was not able to obtain any evidence that heparin combines with prothrombin finding that it did not prevent the activation of prothrombin in a mixture

titative in many experiments. Yet one finds all sorts of inconsistencies and contradictions. For example compare (a) every fibrinogen solution which has been tested has coagulated with calcium chloride alone and the statement (b) but a consideration of the properties of kinase and prothrombin and the constitution of plasma point to the conclusion that calcium alone will not clot fibrinogen solutions. As 'thrombo kinase' Mellanby used diluted testicular extracts from the bled cockerel after the manner of Rauschenbach (1881). With calcium this readily clotted his 'fibrinogen' and he properly interpreted this as evidence for prothrombin contamination in the substrate. In this early work [327] Mellanby did not separate prothrombin but merely deduced its presence and activation from crude experiments especially varying the concentration of kinase.

Mellanby's 1909 thrombin (fibrin ferment) was simply the fluid expressed after complete coagulation of a fibrinogen solution by kinase and calcium chloride. It was with such reagents that he attempted to quantitate the thrombin fibrinogen reaction both as to clotting times and fibrin yields. He varied (a) calcium and other alkaline earth salts (b) neutral salt (NaCl) concentrations (c) acid (HCl) or alkali (NaOH) and (d) oxalate citrate fluoride etc.

In 1930 Mellanby [328] presented a purification technique for 'prothrombase' which was his term for prothrombin based upon the prevailing enzyme idea. Starting with dilution and acidification patterned after the earlier methods of A. Schmidt and Pekelharing and essentially anticipating what we would now call an effort toward isoelectric precipitation he triumphed over many difficulties. Thus the precipitate obtained from the diluted plasma is a complex mixture. The greater portion of it consists of prothrombase, fibrinogen and serum globulin but the pigments of serum, cholesterol and thrombokinese (1) are always present in variable quantities. The separation of prothrombase from this complex precipitate has presented considerable difficulties. Nevertheless by diluting with lime water and bubbling CO_2 through to pH 6.8-7.0 and calcium bicarbonate (thus formed) about 0.0092 per cent he claimed splitting of the prothrombase from the complex. The amount of calcium was said to be too small to activate the prothrombase in the time required for filtration and subsequent precipitation by acetic acid to pH 5.3. The final product was dried with acetone (cf Howell see p. 10). Admissions of its frequent contamination with thrombin conflict with assertions of no spontaneous activation e.g. on dialysis. Mellanby's product did have considerable potency and in some tests was partly stable to boiling for 5 min. at pH 7.8.

Addition of tissue extract alone (thrombokinese) to a solution of prothrombase always causes the generation of a corresponding quantity of thrombase in the course of a few hours. This fact holds good even when the prothrombase has been prepared from solutions containing no calcium salts i.e. oxalate plasma and the replacement of calcium bicarbonate by sodium bicarbonate in the second stage of the process. However minute amounts of calcium have a marked effect on the velocity of activation. Hence Mellanby balked at any final conclusion thus "no definite statement on the place of calcium salts in the reaction may be made until thrombokinese has been isolated in a pure condition."

lipoprotein from lungs [87] the phosphatides are split off the protein carrier and a heparin-protein compound results. This compound in losing its thromboplastic property has acquired a new one: it exerts a markedly anticoagulant effect [83]. The protein moiety of the thromboplastic agent must obviously also be considered. Furthermore the plasmatic cofactor (cf. [242]) has been shown [67 151a] to intervene in the antithrombotic as well as the antithrombin actions of heparin and this requires further explanation. As to the second part of the question: real evidence is still lacking that prothrombin was bound in the first place.

4) Can prothrombin be activated by calcium salts alone? This essential corollary to the Howell theory is seriously questioned by more recent work including data presented in this dissertation in which it will appear from experiments with both Howell type and with newer types of prothrombins that cephalin or tissue thromboplastin play a direct role along with calcium in the conversion of prothrombin to thrombin. Furthermore wholly new factors [363 364] have now become recognized as equally essential in the highly complex thrombin forming reactions. In summary then the Howell theory was too simple and has not stood up to the inexorable pressure of more modern experimental facts.

8 J MELLANBY STUDENT AND LEADER IN THIS FIELD

In 1909 J. Mellanby working in the Physiological Laboratory at Cambridge (England) as George Henry Lewes' student published two [327] noteworthy papers in the field of blood coagulation and in his maturity he returned to this field with further investigations in the 1930's [328 329].

Mellanby first employed fowl plasma as recommended by Delezenne (1897) to prepare fibrinogen by a dilution and acidification technique in principle similar to the techniques of Alexander Schmidt [420 421]. He rejected Hammarsten's [205] salting out method because of baneful influences of salts on proteins and stated: "It is clear that if fibrinogen is a globulin the best method to prepare it is to dilute and neutralize any fluid containing it. This procedure will certainly precipitate all the globulins contained in the fluid used but there has not yet been adduced a certain proof that any globulin other than fibrinogen exists in plasma." As a matter of fact the majority of experimental results point to the conclusion that fibrinogen is the only globulin contained in plasma [327]. This error is not only confounded completely by more modern knowledge (ref. [114]) but was untenable even with the data on plasma and serum proteins available in Mellanby's time. Furthermore it is inconsistent with many observations in his own work. Elsewhere Mellanby cited Pekelharing [373] as identifying prothrombin with 'kinase'. We have noted (p. 8) that this authority did believe prothrombin to be a 'nucleo albumin' of cellular origin but he was very clear that it was a plasma component and quite distinct from the zymoplastic cellular substance of Alexander Schmidt.

Credit must be given to the attempts of the Cambridge 'student' to further the knowledge of blood clotting including his efforts to be quan-

1935 [381]) Many including Alexander [6] and ourselves [147] have come to prefer BaSO_4 . In their 1938 purification of prothrombin by CO_2 (under pressure) elution from the $\text{Mg}(\text{OH})_2$ plasma adsorbate and its subsequent transformation to thrombin by means of calcium and lung extract the Iowa workers were assisted by W H Seegers [435 428]. After establishing his own laboratory at Wayne University Detroit Seegers and his colleagues further purified prothrombin [424 431 425] and thrombin [433] from bovine plasma to give us the most potent and possibly the purest preparations to date. There still remain some questions of trace impurities and it must be remembered that both adsorbents and precipitants yield several other components of the clotting system the presence of which in the final product may call for some critical comment.

B MODERN VIEWS ON BLOOD CLOTTING

The following selective outline of present day (1956) knowledge is presented with slight personal bias but is essentially the consensus of the modern 'group thinking'. All workers agree that many details and perhaps still undiscovered factors await further exploration and that the present 'working hypothesis' useful as it is especially in many clinical applications yet fails to give an adequately detailed answer to the deceptively simple but truly involved questions: (1) why does blood not clot in the normal circulation and (2) why does it clot (a) in thrombotic conditions and (b) when shed? To unravel the complexities we shall try to analyze the clotting mechanism step by step bearing in mind however the inter relatedness and frequent simultaneity or overlap of the several and successive reactions.

1 SKELETON SCHEME OF CLOTTING REACTION

Figure 1 presents an intentionally over simplified scheme. It merely indicates the two 'classical' phases of clotting. The essential and final (second) phase is the conversion of the plasma protein fibrinogen into fibrin clot through the physiological intervention of thrombin. From one point of view this reaction is colloidal namely a change from 'sol' to 'gel' involving many physico chemical considerations including surface electrical charges depending upon polar groups of the reacting molecules and the 'ionic atmosphere' of the surrounding salt rich medium. From another point of view it is enzymatic referring to a specific mode of action of thrombin.

Thrombin is not normally present in the circulating blood. Indeed there are antithrombic factors naturally present to inhibit or remove any thrombin which might possibly form in the blood. The plasma does contain a precursor protein prothrombin. The classical first phase of clotting then is concerned with the conversion of prothrombin to active thrombin. This is particularly complex and will require a searching analysis of what our scheme collectively calls 'activators'. Possibilities of inhibition of the activator mechanisms are indicated in the scheme and will also require investigation. It is questionable whether we can group such first phase inhibitors as 'antiprothrombic' without becoming involved in the old controversies of the Howell theory.

Mellanby's 'thrombase' (thrombin) described in a later paper [329] was obtained by slightly modifying the prothrombase method so as to permit conditions favorable to 'spontaneous' activation.

We have cited the foregoing in some detail in order to emphasize the unsatisfactory state of experimentation with the blood coagulation problem as late as the 1930's. Fibrinogen had come a long way with Hammarsten [205] and Howell [230] but prothrombin and 'kinase' and even Howell's [232] purified thrombin were far short of critical requirements. It is in fact all too evident that most workers preferred to avoid facing the issue of contaminant impurities. Most deductions were made as if these did not exist even if at times this meant a considerable twisting of the experimental results. It would not seem that it was asking too much by 1930 to ensure that fibrinogen preparations were free from all traces of prothrombin. Yet nowhere can we find a product which failed to clot with calcium and tissue extract except possibly in some of the earlier experiments of Howell [230]. Most of the time however Howell only used calcium salt in the control test since according to his theory this is all that is needed to activate prothrombin.

9 MILLS: PRACTICE VS PRECEPT

C. A. Mills of Cincinnati was the other notable authority whose personal interest and exchange of ideas played a role in the present writer's entry into the blood clotting field at that time. Of his voluminous writings [334-337] little will be said in this review. We do recall however Mills' insistence on a critical control of fibrinogen preparations for freedom from prothrombin. Mills used cephalin as well as calcium in the preliminary test. Unfortunately much of Mills' magnificent conceptual approaches ended in data which must be discredited because of the failure to realize in practice what he preached in theory. The critical control was inadequate when most needed to support the interpretation of his experiments. The fact was that Mills [338] obtained clotting in his 'prothrombin free' fibrinogen on adding lung extracts or platelets in the presence of calcium. This led him back to the heterodoxy of a second type of clotting and even to the nomenclature of Wooldridge's [508] 'tissue fibrinogen'.

The refutation of Mills' experiments and conclusions was made in 1934 by the Iowa pathologists Smith, Warner and Brinkhous [442]. In searching for a suitable fibrinogen these careful workers finally adopted the technique of adsorption with $Mg(OH)_2$ for removal of traces of prothrombin as previously recommended by Fuchs [180]. Their second contribution was in the preparation of the lung extract itself. Being highly vascular lung is ordinarily full of blood, the prothrombin from which can appear in crude aqueous extracts. By perfusing the lung with water or saline the Iowa workers were able to obtain a subsequent prothrombin free extract. This did not clot the critically prepared fibrinogen even on adding calcium.

Prothrombin adsorption techniques were not new. Pickering [375] reviewed a variety of reagents which had been tried for this purpose. We may list $Ca_3(PO_4)_2$ (Bordet & Delange 1914 [v 55]), Na_2SO_4 (Dale & Walpole 1916 [98]), $Mg(OH)_2$ (Fuchs 1929 [176]), $Al(OH)_3$ (Quick

uncertainties but have clearly proved their value in many exacting quantitative tests. Particularly significant are data which have been obtained with physical measurements e.g. opacity rigidity and tensile strength of clots light scattering (differential refractometer) streaming double refraction diffusion viscosity electrophoresis ultracentrifugation and electron microscopy. Solubility studies [89] are very valuable in preparative procedures when worked out in relation to the five variables: (1) temperature (2) pH (3) ionic strength (4) protein concentration and (5) content of water miscible organic solvent e.g. ethanol zinc glycine etc. Monumental contributions in these areas of our field have come from the late E. J. Cohn's Department of Physical Chemistry and Plasma Fractionation Laboratories at Harvard [114].

a) Fibrin yields. The modern chemist seeks to follow the course and kinetics of reactions especially enzyme actions by timing changes in the substrate as well as by measuring amounts of the final products. This logical approach is beset with technical difficulties in the case of the thrombin fibrinogen reaction. The extent to which these may be overcome is illustrated in the selected references [159 348 60]. Fibrin yields as measured by the amount of coagulated protein recoverable in washed clots is of course primarily dependent upon the initial amount of fibrinogen available. That the thrombin concentration determines only the rate of the clotting reaction but not the ultimate fibrin yield is clearly shown in Figures 3 and 4. These are from experiments with purified fibrinogen and thrombin in the author's laboratory [60]. Figure 3 shows effects of varying the relative thrombin concentration a thousandfold but employing a constant amount of fibrinogen. Despite clotting time (see below) variations between 18 sec and 30 min the final (10 day) fibrin yields are identical and all 100 percent of the original (coagulable) protein in the fibrinogen. These findings are strong evidence for the enzymatic nature of the thrombin action. Figure 4 shows fibrin yields with time employing four different thrombin concentrations. The linearity of the logarithmic plots (of residual substrate concentrations) within small limits of experimental error indicates a first order reaction [273] again consistent with an enzymatic activity of thrombin.

NOTE: The results do not rule out a pseudo first order reaction in which preliminary phases might be slower than the action of thrombin. Other things which do not affect the fibrin yield within reasonable limits are (1) temperature (2) pH (3) salt concentration e.g. NaCl CaCl_2 (4) 'fibrinoplastic' colloids e.g. acacia (except for minor occlusion effects) (5) minor partial denaturation of fibrinogen (profilin of Apitz 1937). Things which may affect fibrin yield include (a) extremely low fibrinogen concentrations (0.002-0.003 percent) where 'soluble fibrin' i.e. intermediate polymers of insufficient degree of aggregation forms a significant proportion of the end product according

*Ferguson (1940 [134]) proposed a revival of this old term of Alexander Schmidt in order to indicate agents which shorten clotting times through nonspecific effects best interpreted as adsorptive or similar ways of bringing fibrinogen and thrombin 'reactive groups' together and thus facilitating their interaction.

2 EXTENDED SCHEME OF CLOTTING AND HEMOSTATIC MECHANISMS

Figure 2 presents a more detailed scheme of the major factors in blood clotting and hemostasis with some indication of interrelationships. For clarity all inhibitor factors are omitted and must be considered separately. Briefly summarized the following ideas are covered:

- 1) Hemostasis is a physiological function involving:
 - (a) vascular integrity and vasoconstrictor mechanisms
 - (b) platelets (and other formed elements) participating in the cell thrombus
 - (c) fibrin clot
- 2) Vascular factors will not be considered except to mention:
 - (a) vasoconstrictor role of serum serotonin derived (in part) from 5 hydroxy tryptamine of platelets
 - (b) role of damage to vascular endothelium in (i) initiating platelet participation (adherence clumping breakdown) and (ii) affording site for fibrin deposition
- 3) Platelet factors (incompletely reviewed) including:
 - (a) the above vasoconstrictor (2a)
 - (b) a platelet component which reacts with plasma factors in 'thromboplastin generation'
 - (c) a platelet 'accelerator'
 - (d) platelet retractor factor
 - (e) a role of thrombin in the platelet mechanisms
- 4) Plasmatic thromboplastic factors (AHF, PTC, etc.) are indicated as reacting with platelets (3b) in 'thromboplastin generation'
- 5) Injured tissue sources of a 'complete' thromboplastin are indicated
- 6) Prothrombin is the inactive precursor of thrombin in plasma normally activated by Ca^{++} and thromboplastin in the presence of (7) and (8) which are two essential plasma 'co factors'
- 7) Proconvertin yields convertin in the presence of Ca and thromboplastin
- 8) Proaccelerin yields accelerin under the influence of a trace of thrombin
- 9) Active thrombin formed by the indicated reactions
- 10) Conversion of fibrinogen into fibrin 'Serum factor' (p 21) should be included
- 11) Clot retraction subsequently results from action of a platelet factor (3d)
- 12) Fibrinolysis and clot resolution result eventually from activation of the proteolytic enzyme system whose components are also indicated in the scheme
- 13) Various inhibitors should be included in the concepts outlined above (see pp 29-33)

3 THE THROMBIN-FIBRINOGEN REACTION

Most of the early work in this area was poorly quantitative and beset with uncertainties and errors caused by impurity of the reagents used. Modern materials are by no means completely free from all

but show clots on subsequent thawing. This may indicate that traces of thrombin can act on fibrinogen slowly even in the frozen state but it is just possible that the clotting occurs in the act of thawing.

4) pH: There is an ill-defined pH optimum for the thrombin-fibrinogen reaction with some dependence upon the experimental conditions. It is important that the normal pH of the plasma (about 7.4) is close to this optimum. Clotting times are prolonged but only slightly until we approach the extremes of (a) 5.3 in the acid range or (b) 10 in the alkaline region. In (b) we run into the 'reversible inhibitor' problem (see (7)) of insufficient polymerization of the fibrinogen [466]. This may also be a factor in the acid region [405].

5) Ionic strength: The most important action of sodium chloride and most common neutral salts is a delay in clotting times with increasing ionic strength [115]. Numerous data on this go back all the way to William Hewson (1770). Polyvalent anions [191] e.g. ferrocyanides, ferricyanides have especially marked inhibitory effects. Table I summarizes an experiment of the author's [145]. The experiment shows that the inhibitory action of strong neutral salt (4.5% NaCl) which can be removed by subsequent dilution does not alter fibrinogen or thrombin and only prevents the appearance of visible fibrin in a mixture of these two agents. Confirmation of the fact that clotting times become progressively shorter the longer the thrombin-fibrinogen mixture is held with the 4.5% NaCl before dilution is regarded as evidence that the essential step in fibrin formation proceeds independently of the presence of the salt which merely inhibits or retards the gel formation (precipitation) cf. Apitz [18].

6) Specific ion effects: Calcium is anomalous in that it definitely accelerates the clotting time of thrombin-fibrinogen mixtures in a narrow range of low concentrations e.g. 0.008-0.032 molar in one of our experiments although this depends on total ionic strength [115, 310]. By removal of the Ca effect EDTA hinders the fibrinogen-fibrin transition [409].

7) Reversible inhibitors: In an extensive recent study by Shulman [438] 42 (out of 80) substances tested markedly delayed or inhibited clotting in thrombin-fibrinogen mixtures under this author's test conditions. This type of inhibition is reversible by dialysis. Important conclusions as to the 'polymerization' process by which fibrinogen is transformed into fibrin can be drawn from these and other experiments. We shall consider this topic in a later paragraph.

8) 'Fibrinoplastic' colloids [60]: The shortening of clotting times by these agents has been mentioned in the preceding section on fibrin yields and the footnote on p. 17 suggests an explanation of their mode of action. Gum acacia is of some importance in that it is used to stabilize the reactivity of fibrinogen in the Iowa two-stage prothrombin (and thrombin) bioassay [443]. Protamine has similar effects [134] over a wide pH range. We have also noted it with a highly purified lactoglobulin. Also see 'platelets' (p. 51).

9) Partial denaturation of fibrinogen [60]: The present author's view is that the effect of this is due to the same general type of 'fibrinoplastic' action as noted under 8). That all of the denatured fibrinogen is included in the fibrin clot may be evidence of an adsorptive or occlusion phenomenon. The Wohlisch [504] 'denaturation theory' of clotting is now largely discredited.

to Morrison [348]; (b) occluded proteins [348]; (c) reversible inhibitors of the type studied by J D Ferry S Shulman et al in the Wisconsin Laboratories [160 438 440] for the same reason; (d) fibrinolytic enzymes if active which are apt to be contaminants of thrombin and some fibrinogen preparations and can destroy fibrinogen and fibrin at approximately equal rates according to experiments in our laboratory [291]

b) Clotting times The earliest efforts to obtain quantitative information about the clotting process were by measurement of the time required to form a visible or a solid clot. Because so many factors enter into the determination of any 'clotting time' the whole concept would seem to be fraught with extreme empiricism. In fact it is possible to advance the view that there is no such thing as clotting time since the blood does not normally clot in the circulation so that we must merely be measuring the results of highly artificial conditions. In actual practice however these conditions can be defined sufficiently to give clotting time determinations real value in following and interpreting the clotting phenomena. As a technical point particularly referring to experimental systems of artificially isolated clotting agents most workers prefer to time the first appearance of a definitely visible clot (fibrin strands) rather than a solid gel (tube invertible). Both end points are empirical and depend upon a certain amount of fibrin formation by no means indicative of the ultimate fibrin yield. The appearance of successive crops of clots when a weak thrombin is used is a well known phenomenon. Factors known to influence clotting time include

1) Concentration of thrombin: the stronger the thrombin the shorter the clotting time other things being equal. This is a common feature of enzyme reactions. Figure 5 shows an experiment from the author's laboratories in which over at least a 10 fold range of relative thrombin concentrations the clotting times obeyed an 'inverse law' an old idea in the coagulation literature [163]. It is perhaps unfortunately very difficult to define the precise experimental conditions for obtaining this result. In most experiments the deviation from a linear relationship is significant. It may still be possible however to translate clotting times into relative thrombin concentrations ('units' of activity) by use of this type of reference curve (simple plot) obtained under closely similar experimental conditions [20].

2) Concentration of fibrinogen over a considerable range this has remarkably little effect on the clotting time which is not so strange remembering that we time only a partial change in the substrate (see above). The quality of the clot varies however and may affect reading of the end point. Particularly with very weak fibrinogens a poor clot and the probability of slower and less complete polymerization (referred to under fibrin yield) may cause longer clotting times.

3) Temperature: It has long been known that cooling retards the reaction while warming accelerates it up to temperatures where complications appear because of thermal denaturation of the fibrinogen. Thrombin is much more heat resistant but this depends on pH salt content and other considerations. Some plasmas and impure fibrinogens may be completely clear when frozen and kept at 20°C

but show clots on subsequent thawing. This may indicate that traces of thrombin can act on fibrinogen slowly even in the frozen state but it is just possible that the clotting occurs in the act of thawing.

4) pH: There is an ill defined pH optimum for the thrombin fibrinogen reaction with some dependence upon the experimental conditions. It is important that the normal pH of the plasma (about 7.4) is close to this optimum. Clotting times are prolonged but only slightly until we approach the extremes of (a) 5.3 in the acid range or (b) 10 in the alkaline region. In (b) we run into the 'reversible inhibitor' problem (see (7)) of insufficient polymerization of the fibrinogen [466]. This may also be a factor in the acid region [405].

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10) Adsorptive removal of thrombin: Compared with whole blood or plasma clotting (see later) the surface of the container has little effect on the thrombin-fibrinogen reaction. An exception is seen in the case of very weak thrombins where adsorption onto glass etc. removes part of it from solution and hence lengthens the clotting-time. This can be minimized by coating the tube with non-wettable silicone [60]. It is a common experience that poorly washed glassware thus contaminated with a trace of thrombin can clot fibrinogen solution stored therein later.

c) Nature of fibrin formation Progressing from the uncertainties of Apitz [18] Laki and his colleagues [278-274] produced evidence of a step wise process in the formation of fibrin from fibrinogen under the influence of thrombin. The latest interpretation of these and other data was well brought out in the presentations and discussions at the 1951 Conference of the Josiah Macy Jr. Foundation [274]. J. T. Edsall (representing the Cohn laboratories), J. D. Ferry (formerly of the Cohn team but now at Wisconsin), K. Laki (now at Bethesda Md. NIH Laboratories), D. F. Waugh (Massachusetts Institute of Technology) were the chief authorities at this meeting with the present author contributing a minor role. The modern concept of fibrin formation is as follows:

Fibrinogen is a fibrillar protein whose long filamentous molecules approximate 38 Å in diameter and 700 Å in length (usually) with a molecular weight close to 500,000 (perhaps 330,000). In solution these large molecules moving with Brownian movement exhibit considerable swirling and 'steric hindrance'. During clotting however they line up 'like logs in a stream' and unite end to end and also side to side until the 'polymerization aggregates' are large enough and hydrophobic enough to separate from the watery solution as visible fibrin. Fibrin filaments or needles were seen to appear during clotting under the ordinary transilluminating microscope by Ranvier (1873) and Schimmelbusch (1885). Stübel in 1914 and Howell in the same year demonstrated them very clearly with the dark-field microscope. Howell [234] discussed the problem of the fibrin gel formation in 1916. Electron microscopy first used for observing fibrin by Wolpers and Ruska [507] in 1939 has been used to support the modern views by Schmitt [422], by Hawn & Porter [212], Hall [204], Kaesberg & Shulman [264] and others. Even the electron microscope does not resolve the fibrinogen molecules and the simplest polymers or protofibrils but it does show variations in meshwork density and the fibrillar composition together with cross striations of the fibrils which appear uniform under the ordinary (including dark field) microscope. Consideration of clotting conditions (see above) in relation to electron microscopy and many physical measurements (see earlier) affords strong support for the modern concepts. Thus under inhibitory conditions much smaller aggregates are formed and resemble the normal early or lag phase in which there is yet no visible clot but viscosity birefringence, opacity and other data indicate some intermediate polymerization. Under some conditions the fibrin polymer is reversible undergoing disaggregation and resolution e.g. in urea, guanidine lithium bromide (strong solutions) etc. [307]. Irreversible and urea insoluble clots have recently been shown to depend on two things:

namely (1) calcium [266] and (2) a special 'serum factor' by Laki & Lóránd [277] Lóránd [304 305 307] and Shulman [439]. An earlier indication of this was found in 1944 by Robbins [405] in studying the somewhat similar problem of 'solution' of fibrin in acid (0.03% HCl) or alkali (0.5% Na_2CO_3). These data assist in explaining some of the peculiar actions of calcium when present in the thrombin fibrinogen (+ serum factor) mixture (see above) the 'serum factor' being a common contaminant of 'purified' fibrinogen (but see p. 65).

It is suggested below how a limited attack on the fibrinogen molecule activates polar groups which enable the altered substrate molecules to aggregate in the polymerization processes. The electrical charge distribution on fibrinogen molecules has been studied and there is good evidence that Coulomb forces play a role in the preliminary phases of aggregation. Salt inhibitors (see ionic strength) are to be interpreted (Ferry [157]) as 'shielding' of the polar groups by the charged 'ionic atmosphere' contributed by the salt. Further stages involve van der Waal forces (e.g. adsorption etc.) and perhaps specific chemical bonds (currently debatable) [158]. At all events the final steps do not involve thrombin. Like other enzyme systems thrombin is not truly a part of the fibrin end product. It is a practical fact however that a considerable amount of thrombin is adsorbed onto the fibrin clot [434]. Indeed upon this depended the success of early methods of obtaining thrombin by extraction from washed blood clots.

d) Thrombin. This is by no means the simple proteolytic enzyme suggested by the 19th century coagulationists. It is unlike trypsin [458]. Most of the alleged evidence for its proteolytic powers [219 380] can be dismissed as due to contaminant fibrinolysin [149 462] or possibly as a certain degree of solubility of fibrin [247]. However Sherry and his associates [437] have recently shown that fibrinolysin free thrombin can hydrolyze certain bonds in some synthetic polypeptides e.g. TAME (tosyl arginide methyl ester).

Lóránd [307] has contributed some provocative new ideas. He uses Seeger's citrate thrombin (see p. 23) and (a) bovine fibrinogen or (b) human fibrinogen [308] to show

- 1) non protein N is liberated *pari passu* with the clotting action of thrombin;
- 2) the glutamic acid missing from the fibrinogen after the action of thrombin can be identified in a 'fibrino peptide' which has been isolated and characterized [309 306];
- 3) in bovine fibrinogen thrombin is thought to split arginine glycine bonds [30]; (NOTE: It does not do so in the case of insulin according to Middlebrook.)
- 4) alanine replaces glutamic acid in the N terminal amino acid residues of human as compared with bovine fibrinogen but the general process is the same.
- 5) electrophoretic studies show a characteristic pattern and the paper strip method permits characterization of the split products. The pattern with thrombin is quite different from that produced by fibrinolysin.

Lorand concludes that

- a) the 'fibrino peptide' is a by product which does not participate in the fibrin polymerization but does account for the

10) Adsorptive removal of thrombin: Compared with whole blood or plasma clotting (see later) the surface of the container has little effect on the thrombin-fibrinogen reaction. An exception is seen in the case of very weak thrombins where adsorption onto glass etc. removes part of it from solution and hence lengthens the clotting time. This can be minimized by coating the tube with non-wettable silicone [60]. It is a common experience that poorly washed glassware thus contaminated with a trace of thrombin can clot fibrinogen solution stored therein later.

c) Nature of fibrin formation Progressing from the uncertainties of experiments of Aptis [18] Laki and his colleagues [278-274] produced evidence of a step-wise process in the formation of fibrin from fibrinogen under the influence of thrombin. The latest interpretation of these and other data was well brought out in the presentations and discussions at the 1951 Conference of the Josiah Macy Jr. Foundation [274]. J. T. Edsall (representing the Cohn laboratories), J. D. Ferry (formerly of the Cohn team but now at Wisconsin), K. Laki (now at Bethesda Md. NIH Laboratories), D. F. Waugh (Massachusetts Institute of Technology) were the chief authorities at this meeting with the present author contributing a minor role. The modern concept of fibrin formation is as follows:

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of prothrombin e.g. by calcium ions and thromboplastic (thrombokinase) factors with possibilities of inhibitors which have merely been confirmed with the modern 'purified' prothrombins. Contamination with (a) traces of activators (b) traces of inhibitors (c) fibrinolytic enzyme (or precursor etc.) (d) perhaps other factors may still need critical consideration even in the best modern preparations.

b) Nature of prothrombin [425] Prothrombin is a specific protein accompanying the globulin fraction(s) of the plasma proteins. Its quantity in the blood (15 mg/100 ml) [425] is very much less than that of fibrinogen (280 mg/100 ml). In fact unlike the latter it does not show an independent peak in plasma electrophoresis [491]. Seegers and his colleagues have prepared the most potent hence presumably the purest prothrombin to date and their work provides the most reliable data on its characterization [425]. Seegers believes that most of the difficulties in obtaining a biochemically pure prothrombin come from some activation and other marked alterations in the molecule during the purification storage and experimental procedures. The purest preparation examined by Lamy & Waugh [280] was monodispersed in the ultracentrifuge but with some boundary spreading. Its physical constants led to computation of the molecular weight at 62 700 and the shape of an ellipsoid molecule of $119 \times 34 \text{ \AA}$. Chemical analysis yields a trace of S (cystine and methionine) some 4.3 percent polysaccharide carbohydrate [431] and 3.75 percent ash. Perhaps because of 'derivatives' (Seegers) prothrombin solutions show more than one electrophoretic component but the major component constitutes 70-90% of the total [432]. The thrombin yield may depend upon changes during storage in the dry (lyophilized) state but generally averages 23 000 units* (two stage assay) per mg tyrosine or 1400 units/mg dry weight. Highest: 32 300-35 800*.

c) Biophysics of thrombin formation [280]

(1) Biothrombin is a convenient term [280] for the thrombin obtained from purified prothrombin in the conventional manner i.e. addition of calcium salts tissue thromboplastin accelerator globulin (pro)convertin etc. Maximal transformation to thrombin is obtained only when the prothrombin concentration is relatively low. For physical studies concentrations of prothrombin of the order of 0.5 percent must be used (about 10-100 times as concentrated as used to obtain maximal activation). Under these conditions not more than 50% of the 'maximal' thrombin yield can be secured. With the (lung) thromboplastin allegedly removed by high speed centrifugation subsequent ultracentrifugation shows a single symmetrical peak having an average sedimentation constant $S \sim 4.8$ corresponding to that of the original prothrombin. Some boundary spreading suggests polydispersity however. The specific activity of the material at the peak was 30 000 (thrombin) units/mg tyrosine.

(2) 'Citrate thrombin': distinguishes a new product which Seegers et al. [432] obtained in 1948 simply by incubating purified prothrombin in 25 percent sodium citrate. Several other salts (but not all) could be used and the reaction could be modified by certain diphenyl sulfones [432]. It is extremely difficult to evaluate the biological significance of this very artificial method of thrombin formation. However it must have biochemical significance. Refractive index ultracentrifugation

small quantity (about 2%) of nitrogen not recoverable in fibrin even in optimal clotting of the highly purified fibrinogen now available

- b) the fibrinogen molecule activated by this action of thrombin now contains some 20-30 acidic groups which can very well account for the first phases of polymerization to fibrin
- c) with prolonged action of strong thrombin e.g. 3-4 mg thrombin acting on 150 mg fibrin for 24 hours at 37°C complete fibrinolysis can occur (Guest and Ware [203]) associated with further small NPN increase [307] Hence
- d) fibrin is just a transitory stage in the continuous degradation process of fibrinogen by thrombin

In this special way therefore we may now return to a proteolytic concept of the action of thrombin on fibrinogen

e) Other coagulants, etc. Properly purified fibrinogen solutions are quite stable and are never 'spontaneously' coagulable. Whereas thrombin is the coagulant always required under physiological circumstances some other means of coagulating fibrinogen are available experimentally. Examples are

- 1) crystalline papain [112] a proteolytic enzyme from the paw paw (papaya) fruit. Unlike thrombin (usually) papain readily goes on to digest the clot
- 2) staphylocoagulase [186] a product of the reaction of a pro staphylocoagulase from broth cultures of certain micrococci with a plasma factor [187] which is apparently unrelated to prothrombin
- 3) certain snake venoms e.g. 3 species of Bothrops and 3 or 4 of Crotalus according to Eagle's studies [111]

True fibrin clotting should be distinguished from a variety of fibrinogen precipitations (which are reversible) and denaturations (most of which are not). The pseudo clot with ninhydrin [82] is a case in point. Some of the author's [155] dark field observations of (a) thrombin (b) papain and (c) ninhydrin clots are shown in the accompanying photomicrographs (Figure 6). (III) and (IV) show the nondescript appearance of ninhydrin clots compared with the fibrillar gels formed with (I) thrombin and (II) papain. Laki [275] has recently confirmed the last

4 THE CONVERSION OF PROTHROMBIN TO THROMBIN

It is with this area of the clotting field that the major investigations of this dissertation are concerned. The best modern prothrombins do not change into thrombin 'spontaneously' (but cf [425]). Among the activators mentioned in the schemes of Figures 1 and 2 are: (1) calcium (2) thromboplastic factors (3) 'accelerator' factors (4) 'convertor' factors. Noncommittal terms are used in approaching these problems with an open mind.

a) Purity of prothrombin preparations. Prothrombin is difficult to purify. The early preparations of Schmidt, Pekelharing, Hammarsten, Mellanby, Howell, Eagle [110] and others were undoubtedly too crude when reviewed in the light of modern advances [425, 276, 491]. Nevertheless they gave us many of the basic ideas concerning the activation

have continued to suggest that (protein) 'bound' calcium can play a role in clotting. Even Quick [383-394] still retains the idea that prothrombin is a 'calcium compound'. There is little point in arguing about this, since the weight of evidence supports the conclusion that ionized calcium must participate at some point in the conversion of prothrombin to thrombin. A possible reconciliation of the two view points will be suggested from data presented in this dissertation. Under all ordinary conditions of activating purified or even crude prothrombins, omission of ionized calcium salt or the presence of sufficient excess of citrate or oxalate fails to result in thrombin formation by thromboplastin (and adequate accelerator and convertor factors). The special conditions under which thrombin may be formed apparently in the absence of ionized calcium include (1) Seegers' [432] 'citrate thrombin' formation (see above) which we shall not attempt to explain; (2) experimental mixtures such as those of Milstone [339] (see p. 48) and of recent workers [40] with the thromboplastin generation test (see p. 48) which are best explained as 'intermediates' containing bound Ca (which however was ionized in an earlier phase).

f) Thromboplastin and the activation of prothrombin. This topic will be explored in much detail in the body of this thesis. Aqueous (saline) tissue extracts of many kinds are powerful activators of purified (and crude) prothrombins in the presence of calcium salts and adequate amounts of 'accelerator' and 'convertor' factors. Purified cephalin seems to have thromboplastic actions, but much weaker especially with Seegers' and similar prothrombins. It is now generally regarded as an 'incomplete' thromboplastin. Platelet suspensions also act as an 'incomplete' thromboplastin. So does a red cell lipid fraction [105]. However, when platelets or cephalin are incubated with several other plasma factors (including AHF, PTC, etc. see later) a 'complete' thromboplastin is 'generated'. Modern workers are still trying to find full explanations for these experimental facts (see p. 47).

g) Thromboplastic enzyme. The coagulant property of weak solutions of pancreatic trypsin, which some earlier observers erroneously concluded to be a 'thrombin-like' action, was clearly shown by Eagle & Harris [112] to be the result of some role in the activation of prothrombin in experimental test systems. Some of the author's experiments with crystalline trypsin will be mentioned in this thesis. More recently, Schultze & Schwick [423] have examined extensively the conditions under which trypsin transformation of prothrombin to thrombin can be carried out. Lamy & Waugh [280] report some ultracentrifuge studies (see 'trypsin thrombin' p. 24).

The author [141] spent several years trying to establish a similar role for the natural plasma 'trypsin' (fibrinolysin or plasmin) and possibly to give it an important role in the initiation of ordinary blood clotting [136]. However, the final outcome [476] was evidence that trypsin inhibitors could prevent the proteolytic actions of fibrinolysin without removing the 'thromboplastic' effect, quite contrary to the findings with trypsin. Pending possible reopening of this line of investigation, it must be concluded that any thromboplastic effects of fibrinolysin preparations must be due to some unidentified impurity which they contain. Ratnoff, Hartmann & Conley [402] also investigated the

and electrophoretic studies on 'citrate thrombin' give evidence of an initial splitting of prothrombin into two similar (molec wt : 34 000) fractions but on dilution there appear 'aggregates' and 'disaggregates' and continuing changes over a 24 hour period [280]

(3) 'Trypsin thrombin' is also discussed in the cited reference [280] and will be mentioned again later (p 25)

These modern data are difficult to analyze but do seem to indicate that prothrombin can split up into a number of molecular fragments some of which can re aggregate probably into new agents Autoprothrombin I has been identified with proconvertin [491 425 13] while autoprothrombin II has properties resembling PTC [429] In the formation of both 'biothrombin' and 'citrate thrombin' small but significant amounts of CCl_3COOH soluble carbohydrate and tyrosine compounds are liberated confirming the idea that thrombin forms from prothrombin as the result of some 'splitting' process [427] These very latest ideas cannot yet be integrated with the older data on mechanisms of prothrombin activation from the viewpoints which are presented in this dissertation

d) 'Prothrombinogen' (?) In 1949 Quick & Stefanini [396] claimed to distinguish between 'free' prothrombin and a precursor form for which they suggested the name 'prothrombinogen' They compared their experiments with older data of Nolf [351] and Bordet [55] and concluded that their prothrombinogen differed from Bordet's 'proserozyme' in only a few particulars Only a part of the plasma prothrombin is believed by Quick to be in the precursor form and Quick & Hussey [389] (cf [154]) have recently attempted to explain the normal 'prothrombin time' of infants (see our Table LII) on the basis of a lack of prothrombinogen Few contemporary coagulationists consider that Dr Quick has enough factual evidence to support his shrewd ideas (see Macy Foundation Conferences) but Ware [491] is now inclining toward the prothrombinogen idea The present author [154] remains unconvinced

e) 'Calcium in relation to the conversion of prothrombin into (bio)thrombin' Older plasma clotting experiments by Nordbo [352] and McLean & Hastings [325] extended the earlier idea of Sabbatini [417] that clotting depends upon calcium ions There is a definite optimum of calcium required approximately that available in the blood under physiological conditions Not merely the calcium ion concentration but the actual ion 'activity' (in terms of the Debye Huckel theory) requires consideration Nordbo particularly studied this and further pointed out that the 'available' calcium ions in a mixture of plasma oxalate (or citrate) and added calcium salt depend upon a time consuming process of attaining an equilibrium of the different forms (free Ca^{++} inactive (CaCit) anion and protein bound Ca etc) It is because of this time factor that a several fold excess of citrate or oxalate must be added to prevent clotting and conversely that much less than the stoichiometric equivalent of calcium to anticoagulant need be added to restore clotting (cited [125])

Protein bound calcium is present in the blood plasma normally and in prothrombin and other materials prepared from it unless very modern techniques of complete decalcification (e g by ion exchange resins) are used to obviate this A number of workers (cited [125])

the same factor which (a) Warner Brinkhous & Smith [497] suggested as a prothrombin 'convertibility' factor and (b) Mann et al [319] called 'co thromboplastin'. We prefer to adopt the names proconvertin (for the precursor) and convertin (for the activated form) as suggested by Owren [364]. There are other suggested synonyms (p 20 of ref [453]).

Graham & Hougle [198] report that mixture of the bloods of Alexander's original case R [9] and ours R.S. [298] results in mutual correction of the coagulation defects. Together with other experimental findings this suggests that there may be more than one factor involved under the terms SPCA proconvertin factor VII stable factor etc. Calcium and thromboplastin convert proconvertin to convertin and abolish the lag phase in prothrombin activation to thrombin [295]. Serum often contains much unaltered proconvertin as well as some convertin the total activity being much elevated.

In deficiency of proconvertin* prothrombin conversion is very slow and inadequate. Restoration by partly purified preparations from human [9] or bovine plasma (our laboratories) completely restores both rate and yield of thrombin formation. Dr Lewis and the author [298] reported two cases of severe congenital 'hypoproconvertinemia' including Stuart in whom the prothrombin consumption was reduced. This had not been observed in earlier case reports [9] due we believe to their being a milder degree of this defect cf [198]. We have two more recent cases of VII lack (unpublished) in whom the prothrombin consumption was within normal limits. Our congenital 'hypoproconvertinemias' did not respond to vitamin K₁ unlike the milder acquired hypoproconvertinemias accompanying hypoprothrombinemias in (a) vitamin K deficiency of infants (b) liver disorders or (c) after the drugs dicumarol tromexan phenylindanedione etc (author's data) confirmed by Naeye [350]. The one-stage [101] assay method is satisfactory for clinical use [298]. We [298] have also had some success with a two stage technique which enabled us to compute a 'proconvertin' index (see p 51). *Stuart factor was not separated however.

j) Plasma factors in thromboplastin generation. While tissue thromboplastin may very well play a significant role when blood is shed over injured tissues it cannot explain the good clotting which occurs in blood which has been obtained directly from a blood vessel e.g. by venepuncture with every precaution to avoid tissue contamination. The necessary thromboplastin is generated in the blood itself by a complexity of reactions involving (1) platelets (2) several plasma factors which are present normally but deficient in hemophilia and some recently identified 'hemophilia like' conditions (3) some of the other prothrombin activators. The platelet factors and the thromboplastin generation test will be discussed in a later section.

(1) Hemophilia and related problems. Only a few key points will be reviewed from the extensive recent literature in this field and most of the older work will be excluded.

(a) Hemophilia A. Notwithstanding efforts to account for the hemophilic's clotting difficulty on the basis of some inhibitor e.g. the 'antithromboplastin' idea of Tocantins et al [470 474 475] the major accumulation of evidence points to a specific clotting factor present

relationship between proteolytic activity of plasma and blood coagulation and in a later paper Ratnoff & Colopy [401] suggest that they may have been dealing with 'Hageman factor' (see later under Hemophilia)

h) 'Accelerator' factors in prothrombin conversion Many modern workers were fully aware that calcium and thromboplastin did not provide a sufficient answer to the question of prothrombin conversion. The most significant step however came from the very extensive studies of P. A. Owren [361] on a human patient suffering from a peculiar bleeding disorder which he named 'parahemophilia' [362]. The new factor which this patient was finally shown to lack was called 'factor V' and later 'proaccelerin' by Owren [364]. Quick [384] had earlier evidence for a 'labile factor'. Another lead was the 'thromboplastin cofactor' of Fantl & Nance [119]. Owren [361] also provided evidence for conversion of his 'factor V' (proaccelerin) into a 'factor VI' (accelerin). Ware & Seegers [493] prepared the new agent under the name 'accelerator globulin' by which it is now widely known or 'AcG' for short. They also found differences between 'plasma AcG' (proaccelerin or factor V) and 'serum AcG' (accelerin or factor VI) the latter being formed from the former by a trace of thrombin. Stefanini & Dameshek (p. 20 of ref. [453]) list other possible synonyms.

The present author and his colleague Dr Jessica H. Lewis have studied (1) cases of congenital hypoproaccelerinemia [297]; (2) differences between proaccelerin and accelerin in the activation of prothrombin (cf. [492]); (3) destruction of AcG by fibrinolysin [299]; and (4) its role in performance of the two-stage prothrombin assay and in determining prothrombin consumption in clinical cases [153]. In summary:

- (a) (pro)accelerin is essential for the conversion of prothrombin to thrombin by calcium and tissue thromboplastin;
- (b) a trace of thrombin converts the precursor form (proaccelerin) into the active accelerin;
- (c) AcG has more effect upon the thrombin yield than upon the rate of activation which is somewhat delayed when AcG is very inadequate however;
- (d) satisfactory assay methods are now available both by a 'one stage' (specific) technique [395] and a 'two stage' [493] including modifications (see pp. 58-59) developed in the author's laboratory for application to clinical cases of hemorrhagic disorder. Plasma, serum and platelets may be tested by these techniques;
- (e) serum except from the ox, cat or rabbit [349] is apt to be devoid of this factor because of lability and due to the fact that it is used up during clotting;
- (f) AcG levels fall after hepatectomy [322] or liver injury [461].

i) 'Convertor' factors in prothrombin activation The discovery of this factor also owes much to the first clearly identified clinical case of B. Alexander and colleagues [9]. They named the factor SPCA (serum prothrombin conversion accelerator) and obtained evidence for a precursor (pro SPCA) [118]. Owren was on the track of this additional factor [360-366] but it was Koller (et al. [271]) who correctly reinterpreted Owren's data and named it 'Factor VII'. It is probably

and (3) others (see below). Another interesting recall is the obtaining of an 'antihemophilic globulin' from serum (rather than plasma) by the Dutch workers Bendien and Van Creveld [39] a year or two ahead of the Boston (Thorndike) group [368]. Unlike AHG PTC persists in the serum since it is not completely consumed in clotting. The present author suggests therefore that the Dutch workers were really dealing with hemophilia B and that their 'globulin' was a PTC preparation.

(c) PTA deficiency (Hemophilia C). R. L. Rosenthal and co-workers [413 412 414] have uncovered cases allegedly lacking a third plasmatic thromboplastic factor (PTF) which they call plasma thromboplastin antecedent (PTA). We have confirmed the defective prothrombin consumption in one of their cases.

(d) 'Fourth factor' deficiency (Hemophilia D)? Spaet, Aggeler & Kinsell [446] encountered another type of case with a possible fourth plasma component but did not rule out an inhibitor of PTC.

(2) 'Hageman Factor'. Ratnoff & Colopy [401] observed three patients with prolonged bleeding times but without significant hemorrhagic symptoms. Their studies suggest yet another factor, the chief action of which is failure compared with normal plasma to accelerate the clotting of platelet poor plasma obtained for normal blood centrifuged at high speed without anticoagulant. They gave it the name 'Hageman factor' after the first patient and obtained correction of the defect with a fraction prepared from normal BaSO_4 adsorbed heated serum.

(3) 'Factor X'. Duckert, Koller and colleagues [108] investigating sera from various pathological conditions in the thromboplastin generation test concluded that still another factor must be recognized ('Factor X'). It is said to be deficient particularly in sera from cases with hepatitis or persons under treatment with a dicumarol derivative (Marcoumar). 'X' is now used for Stuart factor (see SUPPLEMENT).

5 INHIBITOR PROBLEMS

a) Inactivators of thrombin. Quoting Seegers [425] the following mechanisms participate in the removal or neutralization of thrombin (the comments are those of the present reviewer):

- 1) Thrombin may be adsorbed on fibrin. Fibrinolysin can release it [268].
- 2) Large amounts of thrombin are neutralized by antithrombin. This antithrombin does not require heparin for its action. This is the classical antithrombin of serum or plasma [505 506]. It acts over a period of time in a progressive manner [190] and is limited in degree i.e. a given amount of antithrombin can neutralize up to a definite limit of thrombin. Morawitz [344] used the term 'metathrombin' for the inactive product which thrombin forms with the antithrombin of serum. His claim for partial reactivation by treating serum with acid and alkali was confirmed by Gasser [184]. Astrup & Darling developed a method of assaying antithrombin [21] which they later [22] distinguished from the 'thrombin inhibitor' which heparin forms with some labile plasma component (co inhibitor).

in the plasma of normal persons but deficient in the hemophillic Patek & Stetson [368] and later other workers in F H L Taylor's group at the Thorndike Memorial Laboratory of the Boston City Hospital contributed convincing evidence for this antihemophillic factor (AHF) which they called 'antihemophillic' globulin (AHG) Koller [270] lists it as 'factor VIII' AHG accompanies fibrinogen etc in the Harvard Laboratories' plasma 'Fraction I' It is somewhat unstable and difficult to isolate and purify Bidwell [46] in England claims a good bovine plasma fractionation but the problem needs further solution [488]

AHF is utilized or consumed in the clotting process With other factors it participates in thromboplastin generation from platelets (or cephalin) (p 46) In the natural clotting of hemophillic blood or recalcified hemophillic plasma the 'prothrombin consumption' is very defective Following this lead of Dr Brinkhous's we have also developed a method depending upon the prothrombin consumption (1 hr at 37°C) in recalcified mixtures of 'substrate' (known severe hemophillic) and normal (vs test) plasma for quantitative estimation of plasma AHF levels These tests can also be modified to assay anti AHF [152] By changing the substrate (known cases) they also assay PTC and anti PTC etc

We shall say nothing about the important familial aspects of hemophilia except to mention the very interesting work on canine hemophilia [197]

(b) PTC deficiency (Hemophilia B) In February 1952 Dr Lewis and the author [294] were investigating a 'bleeder' who seemed to be made worse instead of better by blood transfusions Our tests immediately showed that he had a circulating anticoagulant (see later) but by preparing plasma fractions free from this inhibitor an underlying clotting defect was disclosed which was definitely not hemophilia By withholding transfusions the anticoagulant gradually diminished as shown by the tests illustrated in Figure 7 clearly revealing evidence (the long recalcification clotting times in the last tests) of the basic clotting defect In April 1952 Aggeler and colleagues [5] in California published the first clearly identified case of the new 'hemophilia like' disease which they named PTC deficiency (plasma thromboplastin component) Using their techniques we quickly confirmed that our case was the identical disorder but with a new type of inhibitor complication [294] A few months later Biggs Douglas Macfarlane and colleagues [51] published an English case and from the name of the patient coined the term 'Christmas disease' Koller's name for PTC is 'Factor IX' [270]

An earlier lead was provided by a number of observers particularly the Argentinian Pavlovsky [370] namely that the mixing of two 'hemophillic' bloods from certain cases resulted in correction of the clotting times both in vitro and in vivo Very interestingly some of the classical 'hemophillic' families (e.g. Von Tenna) extensively studied in Europe have turned out to be not what has recently been accepted as hemophilia but the similar PTC deficiency (or Christmas disease) To avoid the confusion which has resulted Dr Koller [270] of Switzerland has proposed a revision in nomenclature namely (1) Hemophilia A (the usual type); (2) Hemophilia B (PTC-deficiency);

c) Heparin co factor (heparin complement) [242] Fitzgerald & Waugh [164] have recently fractionated co factor from Cohn's plasma 'Fraction I'. Their tests extend the work of Astrup & Darling [22] and do not support Klein & Seegers' [268] idea of interference with the thrombin fibrinogen interaction (also Glazko & Ferguson's [190] 'immediate' antithrombotic action) but rather point to a definitely time consuming reaction. They conclude that heparin plus cofactor inactivates clotting by removing thrombin by direct combination. Thus the reaction is 'progressive' and 'limited'. Further complications arise since reaction velocity, specific capacity of cofactor (units T/mg C) and extent of reversibility (using protamine) are functions of cofactor concentration. These authors [164] suggest that cofactor does not occur as such in plasma but that heparin cofactors in effect represent altered normal antithrombins.

Some of the author's work with heparin and cofactor etc will be presented in this thesis. We shall not review the chemistry of the heparins nor the modern work on paritol, treburon, thrombocide, sulfonated dextrans and other synthetic heparin like substances of current interest particularly in the search for a cheap heparin substitute.

d) Specific inhibitors of individual clotting factors. These factors being protein in nature for the most part 'antibodies' to individual clotting factors considered a priori are a possibility which recent investigations are beginning to support with experimental facts. Such inhibitors are particularly significant when they can be demonstrated and shown to contribute to the bleeding tendency in clinical cases [453].

(1) Antiplatelet factors causing thrombocytopenia and interfering with clotting and hemostatic functions are discussed on p. 37. Normal and pathological factors which specifically inhibit one or other of the various components of the thrombin forming system are beginning to receive serious consideration as follows:

(2) Anti AHF and anti PTC. The present author and associates [152] presented a paper at the September 1955 Meeting of the American Physiological Society and a definitive publication will appear shortly. By use of the prothrombin consumption test on specifically deficient substrates (lacking AHF or PTC respectively) to which mixtures of normal and patient plasmas were added we assayed the anti AHF and anti PTC inhibitors in eight cases with hemorrhagic disorders. The routine tests on these cases showed prolonged clotting times but normal prothrombin time (Quick test). In five cases with primary hemophilia the anticoagulant could not be demonstrated by simple clot delay on mixing with normal blood or recalcified plasma but required the specific anti AHF test. One hemophiliac bled to death in 18 days following a tooth extraction despite a record 400 transfusions with whole blood plasma and antihemophilic globulin. We could demonstrate removal by the potent inhibitor of the transfused AHF faster than it could be supplied. In all five hemophiliacs the acquired inhibitor was specifically anti AHF whereas in the two PTC deficient cases it was anti PTC. The eighth case of 'idiopathic' in a female and showed a very high titer of anti AHF as well as an apparently high titer of anti PTC. The last might be questioned on technical considerations.

Inhibitors of the above type have been reported in a very small minority of cases of hemophilia, PTC deficiency, certain allergies; some

They state [22] while normal antithrombin seems to combine with thrombin to form an undissociable compound the compound between thrombin inhibitor and thrombin as well as the compound between heparin and thrombin coinhibitor seems to be highly dissociable. That fat solvents can inactivate antithrombin was noted in the early literature chloroform especially being used for this purpose [351] although it has the disadvantage of also destroying thrombin [476]. Seegers et al [433a] have done many experiments with ether especially to remove this antithrombin from defibrinated plasma. Sternberger [456] used alcohol and has obtained some very provocative results [457]. Fibrinolysin will not work [476].

3) Heparin co factor together with heparin interferes with the reaction of thrombin and fibrinogen. This will be discussed further in a subsequent paragraph (p. 31).

4) Antithrombin accelerator activity arises during the clotting process and also neutralizes thrombin. Seegers' [425] new agent (AA = antithrombin accelerator) refers to the antithrombin demonstrable after ether extraction of defibrinated plasma. To it is attributed the disappearance of thrombin formed therein after activation of the prothrombin by calcium (lung) thromboplastin and (platelet) AcG. The latter reagents not being the source of the alleged AA factor. Further (Seegers [425]): There is an inhibitor of the antithrombin accelerator action which we call antithrombin accelerator inhibitor. AA inhibitor is removed by BaCO_3 adsorption and is recoverable in the citrate eluate. These new ideas of Seegers are highly provocative particularly in regard to his suggestion that AA inhibitor is proconvertin or associated with proconvertin in partially purified materials.

b) Heparin [242 382 151a 151 22]. It is now generally agreed that little if any heparin is present in the circulating blood normally [249 61]. The protamine titration method [250] of measuring 'heparin like' activity in plasma is subject to questions of interpretation but does agree with anticoagulant tests e.g. prolongation of clotting-times of whole blood [15] or recalcified plasma routine in author's laboratory p. 61 when heparin is known to be present. This is true both in vitro and in vivo. In vivo however heparin may be removed from the circulation by (a) renal excretion [254] (b) metabolic alteration or (c) a heparinase enzyme [248 252]. Chemical extraction and assay of the metachromatic color reaction of heparin with azure dyes are the most convincing methods [342]. They have recently been used in conjunction with paper chromatography [36]. Wilander's work [501] concerning the origin of heparin from the tissue basophils or Ehrlich mast cells is now generally accepted. These mast cells appear to break down and release significant amounts of heparin (proved conclusively by chemical extraction) in certain pathological conditions particularly those involving an anaphylactoid type of reaction [255]. They also yield histamine [195] which probably forms a complex with heparin [403]. Rocha e Silva [407] implicates a fibrinolytic enzyme activation and finds histamine release to be an accompaniment. Hyperheparinemia can cause bleeding disorders [448].

- 1) it is a constituent of normal (platelet free) plasma
- 2) it inhibits added cephalin or tissue thromboplastin but requires a special series of minimal plasma dilutions in order to demonstrate and assay its effects
- 3) it is less effective in the presence of wettable surfaces or after the blood has clotted
- 4) it is somewhat species specific and does not affect the thromboplastic action of Russell's viper venom
- 5) it can be concentrated in certain plasma fractions and in lipid rich tissue extracts Both these points were confirmed by Overman [358 359]
- 6) it is increased in cases of hemophilia [475]
- 7) it is also increased in post irradiation hemorrhage [471]

Dr Tocantins is a very able and careful worker but there is now considerable doubt as to the significance of the data under discussion. One group [196] raises many objections including questions as to the control of ionic strengths in the limited dilution experiments.

e) Summary of the inhibitor problems and their clinical significance From the foregoing brief review it is evident that the inhibitors are a little known and difficult area in the coagulation field. More knowledge is needed because of their importance in occasional clinical cases. Valid information has begun to accumulate as the result of the devising of specific testing techniques. In some cases the diagnostic question is settled. The therapeutic goal is elusive however. Adrenal cortical hormones failed in an extensive trial on two of our clinical cases. The relationship of inhibitor appearance to transfusions necessitates a searching review of this form of therapy. There is need for more rapid advance in the difficult field of fractionation of the individual clotting factors from the blood in practical preparations which can be used for rational replacement therapy.

BLOOD PLATELETS

A HISTORICAL

Several careful microscopists in the early nineteenth century must be credited with the discovery of the blood platelets although they were ignorant of their true nature and functions. Most of these workers are cited in a 1934 review of the history of haematology by Sir Humphrey Rolleston [408].

Alexander Donne (1842) [106] termed them 'globulins' (a term still used in the French literature) but probably confused them with fatty particles of the chyle. Addison (1841) possibly Wagner (1842) and certainly Andral (1843) [17] observed them in commencing clotting of the blood. Andral thought them to be 'fibrin molecules' as did Fr Simon. G Zimmerman (1848 60) [516 517] also cited Gerber's 'free nuclei' and Fr Arnold's 'Elementarkörperchen' as well as Simon's term 'Kugelchen'. Zimmerman used Simon's technique of receiving blood into potassium ferrocyanide and delayed clotting enough to permit investigation of the supernatant still more or less turbid fluid. He also could receive the blood into neutral or carbonic acid salts. In slowly clotting bloods he clearly noted the 'little bodies'.

systemic collagen diseases [175 453]; and questionably associated with pregnancy. History of previous transfusion therapy can usually be elicited. In one of our PTC cases the evidence was very clear that transfusions led to the recurrence of high titers of the inhibitor. In this case and in the 'idiopathic' female electrophoretic analysis revealed an abnormal increase in serum gamma globulin. This is further evidence that the inhibitor is acquired by some unusual type of 'immunity reaction'. However the result in causing transfusions to become harmful instead of helpful complicates the clinical control of the bleeding problem.

(3) Anti(pro)convertin (Factor VII inhibitor) Wagner et al [487] have obtained certain fractions from normal dog plasma which specifically inhibit active convertin. Jurgens [259] has prepared a similar agent from normal human serum and suggests that it has certain properties in common with plasma and serum antithrombin.

(4) Anti(pro)accelerin (Factor V inhibitor) has been questionably reported by Hürder [223] in a case with a bleeding disorder. It is said to be 'lipoid like' and to cause the factor V deficiency which explains the bleeding syndrome in the case cited.*

(5) Antithromboplastin (A) in the sense of a circulating inhibitor of added tissue thromboplastin in cases with a bleeding tendency is suggested in several accounts referred to on p. 209 of the new text by Stefanini & Dameshek [453]. The present author suggests that the positive identification of a genuine antithromboplastin must fulfill certain criteria:

- (a) It must specifically inhibit added tissue i.e. 'complete' thromboplastin e.g. in the one-stage prothrombin time (Quick) test.
- (b) there should be no alternative explanation possible on the basis of inhibition or lack of some other factor in the thrombin forming system.

Hougie [226] has already pointed out some of the interpretive difficulties. For instance if the inhibition is demonstrable only at high dilutions of the added thromboplastin it might be due merely to lack or inhibition of the plasmatic thromboplastic components (AHF, PTC etc.). In fact the test might be no more in essence than the performing of the partial thromboplastin test of Langdell et al [282]. These authors indeed use it to assay the lack of AHF. When Fantl & Nance [120] investigated an Australian woman and reported inhibition of human brain thromboplastin but not that of other species they might simply have been observing the influence of dilution on two different reagents which did not have true equivalency of thromboplastic potency in the first place.

Among the several cases cited [453] the patient tested by Chaffin & West [81] and later restudied by Conley et al [92] shows that many turn out to be anti AHF, anti PTC etc. AcG controls are important also.

Antithromboplastin (B) in the sense of Tocantins et al [474] refers to an alleged plasma factor for which the following claims are made:

*Ferguson et al described an unequivocal case of anti AcG (*Proc Soc. Exp. Biol. & Med.* 95: 567 1957)

1 PLATELETS IN THE BLOOD UNDER NORMAL AND THROMBOTIC CONDITIONS

Many workers have confirmed and extended the observations of Bizozero [54] and Eberth & Schimmelbusch [113]. Several laboratories have shown fine cinematograph films of the circulation in small vessels including platelet observations e.g. those of (1) M. Knisely [269] (formerly of Chicago now at Charleston S.C.); (2) the research team at the Hoffman-La Roche Swiss laboratories; (3) the Boston University group under G.P. Fulton and B.R. Lutz [41]; (4) Jean Hughes at Liege.

In vivo platelets are tiny little discs which appear lenticular or 'batonnet like' when viewed edgewise. Endothelial injury causes leukocytes and platelets to 'marginate' from the axial stream to the 'skimmed' plasma layer closest to the blood vessel wall. Platelet plugs can be seen to form and seal off microscopic holes in an injured vessel particularly in venules. The platelets adhere individually and to other platelets. They also break away one by one or in clumps of varying size. These may be arrested temporarily or more permanently further along the course of the circulation.

In observations on the formation of cellular thrombi in the frog mesentery on pricking the capillary endothelium with a microneedle (Kite Chambers micromanipulator) the author [127] noted the significance of rate of blood flow. In slower rates of flow all the corpuscular elements enter into the thrombus formation and the red cells being the most numerous largely preponderate. In more rapid rates of flow it is the stickiest elements which have the best chance of remaining adherent. Hence the distinction between 'white' (thrombocyte or platelet) thrombi and 'red' or 'mixed' thrombi long recognized by pathologists. Hayem's (1889) term *clou hemostatique* and the German 'Blutstillenden Keil' are highly descriptive (p. 82 of Quick [383]). Wharton Jones (1851), Mantegazza (1869) and especially Zahn (1872) [513] preceded Bizozero [54] in demonstrating the importance of white thrombi for controlling hemorrhage after vessel injury.

2 VISCOUS METAMORPHOSIS AND PLATELET ADHESIVENESS

The thrombotic platelet mass later evinces a hyaline or granular appearance for which the term 'viscous metamorphosis' was coined by Ebert & Schimmelbusch [113] and later used by J.H. Wright and G.R. Minor [511]. At one of the Macy Conferences [509] Helen P. Wright reviewed this topic and described her quantitative approach based upon rotating the platelet containing fluids in glass tubes for

* Few workers have bothered to check the term *batonnet* as originally used by Aynaud [24]. N. Rosenthal in H. Downey's *Handbook of Hematology* [411] says *baton* like and the latest note of the Boston workers [41] uses *bayonet* like. Actually Aynaud compared this platelet appearance to the little stick with tapered ends which is used in the childish game that the English call *tip cat* the Americans *mumbly peg* and the South Africans *kennetjie*.

(kleine Körperchen) before fibrin appeared and described the latter as forming homogeneous masses or threads often radiating from the 'little bodies' or in some cases from leukocytes. He concluded in favor of the idea that "these little bodies already exist in the circulating blood". As they resisted ether and warming Zimmerman showed they were not fatty globules but an organic (vesicular) formation ('organische Bildungen mit Inhalt gefüllte kleine Bläschen'). Zimmerman also studied birds and amphibia and described the nucleated cells (distinguishable from leukocytes but confused with erythrocytes) which were termed 'thrombocytes' by later writers [100]. He discussed their formation in terms of Schwann's "cell theory" and suggested a lymphatic origin.

In 1865 Max Schultze again noted them in human bloods as did Vulpius (1872) and Riess (1872) who thought they were remains of disintegrated leukocytes. In 1874 William Osler [357] gave another classical description which distinguished them from bacterial microorganisms. In 1877 Hayem [213, 214] called them 'hematoblasts' or the 'third' (formed) element of the blood and insisted that they were precursors of the erythrocytes, a view which he maintained as late as 1923 [216]. While much of the confusion of platelets with the other formed elements may be blamed upon poorly controlled observation in vitro, two more recently identified phenomena may be suggested as sources of error: (1) living leukocytes can lose fragments during observation (dark field) on glass slides in a shed blood drop [122]; (2) erythroplastid [116] formation can occasionally be observed under similar conditions from certain erythrocytes or their precursors.

Figure 8 shows one of a series of 14 photomicrographs taken by a former colleague (Dr P. H. Ralph) over a 2 hr period in an observation of an erythroplastid forming from a bone marrow erythroblast in a case of Hodgkin's disease.

Bizzozero's [54] work was most significant. He described platelets in the circulation in the mesenteric vessels of rabbits and guinea pigs and later in the intact bat's wing. He demonstrated their adhesive quality, their participation in thrombi, and their role in the coagulation of the blood.

Lowit [311] tried hard to prove platelets to be mere artifacts, e.g. leukocyte fragments, but Bizzozero's data settled the controversy. Eberth & Schimmelbusch (1886) gave a classical description [113] of experimental thrombosis, including the role of the platelets and of fibrin in the transilluminated mesentery.

J. H. Wright [510] identified the origin of the mammalian blood platelet from cytoplasmic fragments of the megakaryocytes of the bone marrow. He also showed the phylogenetic relationship to the thrombocyte of the lower animals which serves similar physiological functions in these orders. Dr W. H. Howell showed me his beautiful tissue preparations obtained for a 1937 paper [241] in which he stated: platelets represent a solid secretion from a unicellular gland, the megakaryocyte. This is a good way to emphasize the conception of a dual nature of platelets, namely partly like a cellular element and partly as 'amorphous' material contributing to the chemistry of clotting, etc.

and colleagues [45] These Toronto workers inserted a glass chamber between an artery and vein in an experimental animal and observed platelet agglutination and adherence to the flat surfaced glass chamber microscopically The cinematograph film of these observations was re exhibited at the Macy conference (1949) Heparin caused a great reduction or even absence of observable platelet agglutination Dr Best and others [161] believe that some heparin preparations contain impurities which could explain the findings of Copley et al

4 PLATELET ANTISERA PLATELET GROUPS AND THE THROMBOCYTOPENIA PROBLEM

Following the 1905 suggestion of Marino Bedson [33 34 35] injected rabbit platelets into guinea pigs and obtained a platelet anti serum which caused thrombocytopenia and endothelial (capillary) damage when injected into 'non sensitized' animals Ackroyd [1] has recently reviewed the problem of 'platelet agglutinins and lysins in the pathogenesis of thrombocytopenic purpura He includes:

- a) the 'abnormal splenic secretion' theory of Frank (1925);
- b) the efforts of Troland & Lee (1938) and others to obtain spleen extracts ('thrombocytopen') allegedly capable of reproducing the human thrombocytopenic condition in experimental animals;
- c) the demonstration by Evans Duane et al (1949 51) of a 'thrombocyte agglutinating factor' in the sera of some ITP (Idiopathic thrombocytopenic) patients;
- d) several reports that transfused platelets survive for shorter periods in ITP cases;
- e) Harrington's [207] convincing experiments in which by injecting himself with plasma from an ITP case he lowered his platelet count to dangerously low levels and was fortunate to recover;
- f) confirmatory repetition of this extremely carefully conducted by Stefanini et al [454];
- g) Tullis' [478] finding that 9 out of 18 sera from ITP cases caused agglutination and lysis of normal platelets in the presence of complement;
- h) demonstration by both Harrington's [208] and Stefanini's [455] groups that platelet agglutinins could occur in some patients after pregnancy or transfusions; or
- i) even in some individuals who have never been pregnant or transfused [455]; and
- j) the confusion due to demonstrations [451 502] of fall in platelet count following transfusions of normal blood etc

This whole problem is beset with technical and interpretative difficulties but the following conclusions represent current thinking

- 1) antiplatelet agglutinins and lysins occur in humans sometimes without but usually with thrombocytopenic purpura;
- 2) there are platelet 'groups' or 'types' analogous to those encountered with red cells;
- 3) 'acquired' cases of thrombocytopenia often have a demonstrable cause of agglutinin production Ackroyd [1] has made some very clear cut studies of drug induced (sedormid; quinidine)

definite time periods and comparing the platelet count (hemocytometry) with a vaselined-tube control. She reduced 'platelet adhesiveness' by heparin and chlorazole dyes in vitro and by dicumarol in vivo. Conversely adhesiveness was increased after operations or parturition at periods when the platelet count was also elevated and many young forms were circulating. Similar findings were reported for the thrombocytosis following injections of adrenalin or of pyridine. Splenectomy gave similar results but now there was little further effect from pyridine. Moolten & Vroman [343] with an improved technique failed to confirm Helen Wright's suggestion of an increased platelet adhesiveness in hemophilia. Her idea in this disease is also contrary to observations of many other workers. Dr Wright's interpretations included the following (with the present reviewer's parentheses): the factors which appear to maintain the various (formed) elements in suspension are first their surface charges (p. 174 of Tocantins [469]) which are of the same (electrical) sign; and second their constant motion both as a mass and relative to one another. 'It is believed moreover that the surfaces of the formed blood elements carry variable amounts of adsorbed water and that this helps to render them stable in suspension as well as augmenting the viscosity of the blood.'

their surface reactions may be in part dependent on some of the metabolic activities of the cell. The platelet surface may be modified by substances normally (fibrinogen globulin) or abnormally (immune globulins of anti platelet sera) present in the blood. Adhesiveness must also be related to alteration in the wettability or stickiness of injured vascular endothelium [419]. It may be recalled that Roskam [415] collected many data leading to the suggestion that platelets are surrounded by a firmly adsorbed protein film to which many of their properties may be due. That this is not necessarily fibrin or fibrinogen however is indicated by the normal platelet adhesiveness etc. in a case of congenital afibrinogenemia studied by Pinniger & Prunty [376] and confirmatory observations by Alexander et al. [10] and others [296] cited [7].

3 PLATELET 'AGGLUTINATION' (NORMAL)

According to Aynaud [24 25 26 27] platelets suspended in plasma may be agglutinated by a wide variety of substances e.g. gelatine egg albumin peptone gums lecithin heavy metals dyes and surface tension lowering agents such as ricin sod taurocholate and saponin. The peptone plasma experiments were particularly interesting as showing that platelet agglutination could be independent of clotting. Tocantins [469] reviewed many of the older papers dealing with platelet agglutination under various experimental conditions and in numerous clinical disorders. A number of modern workers have restudied some of these problems on well washed (? plasma free) platelets especially aided by the silicone technique (see pp. 67-68).

Copley & Robb [93] observed that washed platelets could be agglutinated by adding plasma or serum especially the latter and also by 'purified' preparations of globulin prothrombin thrombin placental tissue juice and plasma with a high concentration of heparin (!). This last finding was unexpected and in conflict with the data of C. H. Best

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1 RELATION OF PLATELETS TO HEMOSTASIS ETC

As noted previously platelets play a key role in thrombosis and embolism besides acting as a defensive mechanism against blood loss. Pure platelet or 'white' thrombi are relatively rare and the usual thrombus is 'red' or 'mixed' with the bulk and numbers of erythrocytes preponderating. Moreover clotting is initiated about the time (Erkelens' thesis [118]) that platelets adhere and begin to disintegrate. Hence a thrombus is also rich in fibrin which forms a supportive meshwork and buttressing that adds greatly to the hemostatic effectiveness. In the clinical 'bleeding time test' (e.g. methods of Duke [109] or Ivy [244]) defects of platelet or vascular function rather than of clotting function are causative of prolongation of the bleeding. The bleeding time is not infrequently normal or nearly so in cases of hemophilia for instance. Conversely the whole blood 'clotting time' test (e.g. Lee-White method [286]) is often normal in thrombocytopenics and primary vascular diseases.

Since hemorrhagic disorders [453] can accompany (1) vascular abnormalities (2) platelet deficiencies quantitative (thrombocytopenic) or qualitative (thrombocytopathic or thrombasthenic) or (3) coagulation inadequacies it is evident that all three normally share in the defensive hemostatic mechanisms.

Vascular anomalies are beyond the scope of the present thesis. Just how important the contributions of the first two factors may be is emphasized by known cases of afibrinogenemia [7] who lack the essential clotting mechanism. These cases do have bleeding problems but often manage much better than say a severe hemophiliac. May it not be that certain factor(s) of the thrombin forming mechanisms are also necessary for some platelet functions?

Serotonin, Marjorie B. Zucker [518] performed experiments on the formation of the hemostatic plug which appears after cutting a (small) blood vessel in the rat. Noting a frequent vasoconstrictor effect at the time of platelet deposition Dr. Zucker investigated this additional phenomenon and used an ear vessel perfusion technique for bioassay of the 'platelet vasoconstrictor' factor. The consensus of a number of workers [367] on this problem is that disintegrating platelets liberate 5-hydroxytryptamine [399] which reacts with some plasma factor to become 'serum serotonin' (probably 5-hydroxytryptamine creatinine sulfate) previously recognized by Rapport et al [400]. We have some unpublished data showing low serum serotonin (platelet vasoconstrictor) values in cases with hemophilia and other severe clotting disorders. The suggestion (p. 38) is that thrombin (or some factor in the thrombin forming system) is needed for platelet 'release' of 5-hydroxytryptamine.

2 THROMBOCYTES

The thrombocytes serve the hemostatic functions in lower animals e.g. birds, fish, reptiles, amphibians etc. much as the platelets do in mammals [185-189]. That they are not quite as efficient in some ways

thrombocytopenias showing that both drug and specific antibody are needed for the platelet-destroying action;

- 4) complement is involved in the platelet lysis as it is in hemolytic and bacteriolytic immunity reactions

5 IN VITRO 'ALTERATION' OF BLOOD PLATELETS

In 1873 Ranvier [397] observed the microscopic changes of platelets and formation of fibrin in a drop of shed blood. He described alterations in the shape of the platelets and in "the refraction of their granulations". However his most significant observations were: (1) fibrin needles are often seen first at the edges of platelets or platelet debris; (2) platelet 'granulations' occur at the intersecting nodes of the fibrin network

Aynaud (see p 36) made a classical series of studies of platelet alterations in vitro which have largely been neglected by more recent workers in this field. However they formed the chief background for the present author's observations [124] which are embodied in this thesis. With extraordinarily skillful use simply of paraffined equipment the centrifuge and the microscope Aynaud described the sequence of alterations from the original disc or batonnet (p 35) form to the spiculated 'stellate' swollen and granular (disintegration) forms. The controlling factors studied by this worker include (1) surface (2) temperature (3) dilution of plasma (with citrate saline) (4) the decalcifying (citrate etc.) anticoagulants and (5) many experimental additives (see previously)

Fonio & Schwendener [171] used $MgSO_4$ plasma to observe various platelet alterations some of them bizarre

Zucker [518] confirming unpublished observations in the present author's laboratory agrees that even siliconed surfaces which are our best non wettable surface to date fail to prevent but merely retard morphological changes in the platelets held in citrated plasma. The decalcifying ion exchange resins e.g. Amberlite or Dowex '50' do not help. In fact most recent workers find these unsuitable for platelet recovery and prefer the strong Ca binding 'chelating' agent EDTA (sodium ethylene diamine tetra acetate also called Sequestrene or Versene) ref Tullis [479]

$CaCl_2$ was not found to have much influence on the swelling etc shown by washed ($\times 3$) platelets in buffered saline (Milstone [340]). Whether or not thrombin can produce platelet alterations as claimed by Zatti [515] and Fonio [169] is still an unsettled question (Zucker [518]) but is probably true and this may account for some new facts such as low serotonin assays in severe clotting disorders (Dr J H Lewis the author and M B Zucker unpublished data)

B PLATELET FUNCTIONS

For the purposes of this thesis it is not necessary to review some platelet functions (e.g. metabolism; 'platelet loading'; etc [469]). Included are those concerned with hemostasis and allied functions

Character of the clot its firmness rigidity and elasticity depend nearly exclusively on the fibrin concentration and the occurrence or absence of fibrinolysis. They are completely independent of platelets. Fibrinolysis and clot retraction are independent processes. Clot retraction is due to a special function of platelets.

viscous metamorphosis leads to the formation of long strands or pseudopodia of cytoplasm connecting individual platelets or platelet masses. These strands subsequently contract producing a patchy fusion of platelets throughout the clot. As the platelets are adherent to the fibrin network of the clot the contraction of the cytoplasmic strands and the merging of the platelets result in a contraction of the whole fibrin clot with a squeezing out of the serum and a few of the red and white cells enmeshed in the fibrin network.

From the delicate platelet mechanism producing clot retraction it would be expected that the force exerted by the process is very small. By a simple experiment (p 46 [70]) this assumption is shown to be correct; the force (about 19 mm H₂O) is so insignificant that it becomes extremely unlikely that clot retraction serves any physiological purpose in mammalian life. It can certainly not draw together the walls of even the smallest vessels.

In the crab clot formation and retraction take place in the complete absence of fibrin and are entirely cellular functions. It is suggested that the process is a redundant phylogenetic relic of no importance in higher animal life.

A critical review of Budtz Olsen's work will not be attempted here but the present writer would raise some questions e.g.:

- (1) Platelets don't retract clots formed from fibrinogen with thrombin but only with 'thrombin forming coagulant mixtures'. The present author's experience is otherwise.
- (2) Clot retraction cannot draw together small vessel walls. Tocantins [468] who is cited by Budtz Olsen collapsed cellophane tubing by clot retraction.
- (3) Fibrinogen concentration is the chief determinant of size of fibrin mesh and hence of capillary forces. There are many discrepancies between the ineffectiveness of certain agents in modifying Budtz Olsen's clot retraction measurements and data of physico-chemical and electromicroscopy studies which indicate they can greatly alter the fibrillar composition of the fibrin clot.
- (4) Can fibrinolysis be critically ruled out in connection with clot retraction? (See below.)
- (5) Accepting Budtz Olsen's worthy critical review of Glanzmann's (1918) [188] case data on hereditary hemorrhagic thrombasthenia there are contrary to the South African's opinion undoubted cases of platelet anomalies (thrombocytopathias) in which these elements are deficient in some retractor factor ('Glanzmann's retraktosyme'). Such platelets can be isolated and compared with normal platelets shown unable to induce clot retraction in normal platelet free plasma [453]. Such a case will be described in the present thesis.

b) Role of platelets, Budtz Olsen [70] refers to Thackrah (1819) and others whose work clearly indicates that the fact of deficient clot

is suggested by the relative ease of preparing incoagulable or very slowly clotting plasmas from the thrombocyte possessing bloods by careful collection and centrifuging perhaps even without use of anti coagulants. This is very difficult in mammals even with use of anti coagulants because of the ease of platelet breakdown. There are species differences however and equine blood has been used by a number of investigators [24]

3 PLATELETS AND CLOT RETRACTION

a) Clot retraction In 1951 O E Budtz Olsen [70] of Cape Town published an outstanding monograph on this subject with a very extensive bibliography. His theoretical approach may be quoted verbatim:

The blood clot consists of a three dimensional network of fibrin threads. The serum is held in this network by capillary forces. When the serum artificially is pressed out of the meshes the network collapses and does not again expand when immersed in serum due to adhesion between the individual fibers. This can be seen by manipulation of the network under the microscope. The capillary force holding the serum in the clot might conveniently be expressed by this formula:

$$\text{Force} = \frac{2\gamma \cos \theta}{d \cdot g \cdot r}$$

where γ : the surface tension of the serum

θ the angle of contact of the serum with the fibrin which is an expression of the wettability of fibrin

d : the density of the serum

g : the force of gravity

r : the width of the mesh

which is simply the formula for any fluid in any capillary tube. Consideration of the force of gravity by this author is a new contribution.

When the clot is placed in rigid structures like glass tubes gravity tends to draw the serum out of the fibrin network against the capillary forces holding it back; an artificial 'clot retraction' may thus be produced. To avoid this Budtz Olsen designed an ingenious and simple method ('the suspended clot method'). Under constant (37°C) temperature conditions 5 ml of blood (or test mixture) are floated free from the influence of gravity in an inert oil mixture. The serum expressed during retraction of the clot floats to the surface of the denser oil mixture and can be measured. It is shown that the size of the meshes in the fibrin network and therefore the capillary forces holding the serum in the clot depends mainly on the concentration of fibrinogen in the plasma. 'It unavoidably follows that clot retraction is inversely proportional to the fibrinogen concentration of major influence on clot retraction is the volume of red and white cells in the blood. These cells play a passive obstructive role and it is demonstrated experimentally that an almost linear inverse relationship exists between the volume of packed cells and the clot retraction. correction can be achieved by simply adding the haematocrit to the observed clot retraction.' 'Rigid control of the temperature is essential in all work on clot retraction'

in the most recent work [316] to present conclusive evidence to rule out all possibility of traces of active fibrinolysin being present or formed in the test systems. The present author does not insist on 'the fibrinolytic theory' but merely believes that it should be convincingly ruled out.

This being so it is appropriate to consider some peculiar 'retractility' of platelet 'processes'. This will be done in the present thesis especially in terms of lipid water phase relations. It can be stated with fair certainty however that platelets do not extend and withdraw true 'pseudopods' comparable to those in leukocytes etc. Budtz Olsen's data are very weak on this point. This author however may be referred to for a good comparison between physical 'syneresis' of certain gels and the 'clot retraction' phenomenon. The differences are numerous and support the conclusion: 'the fundamental mechanism of the two processes is probably entirely different'.

4 CLOTTING FACTORS IN BLOOD PLATELETS

a) Need of platelets for blood clotting. There was much doubt about the blood platelets being essential for normal blood clotting until Brinkhous [64] using the newly introduced siliconed surface glassware of Jaques et al [251 288] showed that plasma recalcification clotting times and prothrombin consumption (utilization during clotting) were delayed the more the platelet count was reduced by centrifugation. Successful experiments in obtaining a non clotting platelet free plasma will be cited (pp 67 68) in the body of this thesis. The thromboplastin generation test is discussed on p 46.

b) Erroneous ideas. In the earlier literature about what the platelets can contribute to the blood clotting mechanism appear the following:

- 1) platelets act as a complete 'coagulant' for fibrinogen (a) in the presence of calcium by allegedly providing a tissue fibrinogen (Wooldridge [508] 1886; Mills [338] 1930) (b) in still earlier ideas of a similar kind platelets were not separated from the 'white corpuscles' but only from red cells (Mandl 1842; Buchanan 1843 [68] cited by Anderson [16] 1844).
- 2) platelets contain prothrombin (Morawitz [346] 1905 Bayne Jones [32] 1912 Christie Davies & Stewart [85] 1927; Fuchs [177] 1930; Howell [238] 1935 cited by Tocantins [469] 1938). Disproved by Bordet [55] Mills [338] and Eagle [110].
- 3) platelets directly activate prothrombin through their zymoplastic substance (A. Schmidt [420 421]).
- 4) abnormal 'stability' of platelets is the cause of hemophilia (Sahli [418] 1905; Minot & Lee [341] 1916; Morawitz [347] 1925; Howell & Cekada [240] 1926 Christie Davies & Stewart [85] 1927; Fondo [169] 1932). See present thesis. Hayem [216] 1923 suggested that whatever is responsible for the stability of hemophilic plasma also accounts for the stability of platelets in that plasma.

c) Platelet thromboplastic factor (prothrombin 'activator') Bordet & Delange [57] 1912 implicated platelets as one source of their

retraction in association with certain purpuras was known at the beginning of the nineteenth century Hayem [215] in 1896 was the first to correlate poor clot retraction in purpura with definite reduction in the number of blood platelets Among workers who have experimentally reduced platelet counts by centrifuging plasma and carefully correlated with clot retraction data we may mention Tocantins [467]

LeSourd & Pagniez [289] Bordet & Delange [57] Opitz & Matzdorf [356] and others [70] have recorded inability to obtain clot retraction with platelet 'extractives' but only with 'intact' platelets In recent electron microscopy studies Bessis & Burstein [44 43] observed adherence of fibrin filaments only to speculated 'altering' platelets and not to 'disintegrated' forms Glanzmann's [188] suggestion of a 'retraktosyme' (enzyme like factor in platelets) has therefore not gained acceptance until recently when Fonio [170] claimed separation of the outer 'hyalomere' from the central 'granulomere' of platelets by differential centrifugation and the identification of 'retraktosyme' with the hyalomere fraction Very recently 1956 Magalini & Stefanini [316] claim isolation of a 'retractin' both from platelets and from blood-free tissues Their best method is to disintegrate and extract with ether water (65:35) and then deposit in the cold at 20°C Thromboplastic factor is eliminated by heating for two hours at 56°C and subsequent storage at 20°C Tests for vasoconstrictor factor (5-hydroxytryptamine) are also negative They conclude: 'it could represent a lipid substance'

How do platelets bring about clot-retraction? The weakness of the force involved in clot-retraction is stressed by Budtz Olsen as by previous workers While it is usually sufficient to detach the fibrin strands from adherence to glass this may not be the case in some instances Fibrin is more adherent to collodion [220] and Tocantins [467] observed failure of retraction in such vessels However clot retraction is not significantly better in non wettable siliconed tubes Budtz Olsen reviews much of the older literature (e.g. Fuchs [179]) on the influence of the containing vessel's surface on clot retraction Although he cites their paper in another connection (temperature effect) he doesn't include a very interesting experiment of Lampert & Ott [279] which the present writer has frequently reproduced for the benefit of his students Thus if blood is allowed to clot in a wide test tube paraffined down half of its side the retraction of the clot will subsequently be away from the glass and to the paraffin That fibrin is less adherent to paraffin than glass was shown by Fuchs (1931) and many workers have used paraffined vessels in clotting experiments because of its 'non wettability' It is difficult to explain the Lampert & Ott experiment except on the basis that the glass in some way 'activates' the retraction producing factor

Observing certain similarities between retraction e.g. of plasma clots containing platelets and thrombin fibrinogen clots which do not normally retract but can be made to withdraw from the glass tube and 'clump' when known to contain very small amounts of trypsin or fibrin olysins Ferguson & Erickson [149] revived the idea [219] that clot retraction may be related to fibrinolysis This suggestion is not easily dismissed by the arguments of Budtz Olsen whose experiments give no heed to the quantity of fibrinolytic enzyme It may also be necessary

thrombin formation without affecting the quantity produced. However Eagle may not have tested sufficiently small amounts. His weakest cephalin (0.0001 cc of 0.1% suspension) does not quite reach '100%' activation in his Figure 3 [110].

(2) Prothrombin prepared from hemophilic plasma and compared with a similar preparation from normal plasma showed in the presence of a concentrated platelet suspension considerably slower thrombin formation but a normal (100%) eventual yield. With calcium alone Eagle claimed ultimately normal thrombin yields (actually his curves Fig 1 ref [110] show 80-90%) but very slow rates particularly in the hemophilic. Simple plasma recalcification clotting times showed that hemophilic platelets were as good as normal platelets in accelerating clotting in a series of strengths of the platelet suspensions in both (a) normal plasma and (b) hemophilic plasma. However the stronger platelet suspension (both types) worked less well on hemophilic plasma and the weakest platelet strength did not work at all. In comparable experiments cephalin speeded up the clotting of normal recalcified plasmas but had so little effect on the hemophilic (Table III of [110]) as had previously been shown by Mills [333] that Eagle dismissed it as unaccountably ineffective.

Addis [3] had demonstrated in 1911 that hemophilic plasma was not deficient in prothrombin but that its conversion to thrombin was delayed which led him to the hypothetical suggestion of some qualitative defect in the hemophilic prothrombin. Addis prepared 'prothrombin' by a method similar to that later used by Eagle and Howell [238] pointed out that both studies could be subjected to the same criticism namely that their prothrombin contained thromboplastin and that this might be what is lacking in hemophilia. Using his acetone method Howell with Cekada [240] could not find any difference in quantity or reactivity between the prothrombins of normal and hemophilic blood. Howell prepared a plasma thromboplastin and found less of it in hemophilic plasma. Purified preparations (after glycerol extraction) were said to be protein free (ninhydrin and biuret reaction) but Howell could not obtain enough material for conclusive analyses and turned to the study of lung thromboplastin also known to be able experimentally to restore normal clotting times in hemophilic plasma independent of platelets. Howell did think that his plasma thromboplastin might come from platelets and he was able to prepare active thromboplastic material from platelets both normal and hemophilic (p. 19 of ref [240]).

Funk [169] by cold centrifugation in paraffined tubes was able to obtain hemophilic plasma which clotted very slowly (4-8 hours at room temperature) but rapidly on adding washed platelet suspension from normal blood or less rapidly with washed hemophilic platelets.

Other early workers including those we have dismissed under erroneous ideas argued for more stable or less active platelets in hemophilia but it is not profitable to go into this any further. All recent work supports the conclusion that it is not the platelet which is at fault in hemophilia but some plasma factor (AHF) which works with platelets to generate thromboplastin for participation in the activation of prothrombin to thrombin (see p. 46).

a) Platelets and prothrombin consumption. In 1939 Brinkhous [63]

tissue clot accelerating principle or 'cytozyme' and extracted a thermally stable lipid material soluble in alcohol ether and chloroform but little soluble in acetone which agreed with Howell's [231] 1912 identification of the thromboplastic factor as cephalin. Because cephalin was less potent than crude platelet (or tissue) extracts Howell [231] and Rumpf [416] believed the true thromboplastin to be a cephalin protein (i.e. lipoprotein) complex. Eagle [110] also noted that the lipid extract from platelets had only about 1/10 of the original activator potency. Chargaff, Bancroft & Stanley Brown [78] prepared platelets from horse blood and isolated their various lipids. A phosphatide fraction was found to contain a potent activator. They concluded: present methods for the separation of phosphatides are still much too crude to permit the isolation of compounds in a state even approaching purity. It may very well be that a cephalin containing highly unsaturated fatty acids and therefore more soluble in ethyl alcohol is the real activator of blood clotting. As in their later work on lung thromboplastin, Chargaff et al. [78] also noted that the 'defatted' material after platelet extraction was clot inhibitory. In 1945, Chargaff [77] subjected normal human oxalated plasma to high speed (150 min. at 20,000 r.p.m.) centrifugation and lengthened the recalcification clotting times 3x or more. In the following year, further studies [81] added information that the 'pellet' sediment from such centrifugation had thromboplastic properties similar to those of the 'macromolecular lipoprotein' similarly obtained from lung extracts. Flynn & Standley [167] reported to the Macy Conferences (1949) that their confirmatory observations also included microscopic examination of stained films which clearly showed that the pellet obtained by high speed centrifugation of human plasma is composed of platelets and occasionally erythrocytes. Chargaff was mistaken then in identifying a 'plasma thromboplastin' and was really just confirming Brinkhaus' (1939) observations of the effects of platelet removal on plasma clotting.

In 1935 the present author, knowing only of a short note of Haurwitz & Sladek [211] 1928 containing lipid analyses of platelets visited the Research Laboratory of the Children's Fund, Detroit, Michigan, where a group of workers under Dr. I. G. Macy were performing some excellent lipid (and other) fractional analyses of erythrocytes. After preliminary work on some horse platelets prepared for the author by the Mulford laboratories, the Michigan group completed a study on the lipid distribution of human platelets in health and disease [117] shortly after the appearance of Chargaff's work. Sixty-eight percent of the total platelet phospholipid analyzed as cephalin and it was computed that if this were all liberated it could furnish 100 ml of blood with some 5-10 mg of cephalin. This could be very significant in the light of the quantitative clotting data of Spagnol [447] and of Ferguson [129].

d) Platelets and the activation of prothrombin. Eagle's [110] data were as follows:

(1) platelets (like cephalin) can participate in the activation of prothrombin (cf. [328]) to thrombin. Since Eagle's prothrombin activated slowly (about one hour) with CaCl_2 alone, he concluded that platelets and cephalin accelerate coagulation by increasing the rate of

with Dr Douglas and Professor Macfarlane she showed that three components namely (1) platelets (2) $Al(OH)_3$ -treated plasma (AHF factor V) and (3) aged serum (PTC factor?VII) in the presence of calcium interacted to generate this thromboplastin. Samples of the above mixture removed from the pre incubate at minute intervals are added to platelet poor plasma (prothrombin fibrinogen etc) and observed to give progressively shorter clotting times through a period which is noted before they get longer again. This is the thromboplastin generation test of Biggs and Douglas [48]. The final clot timing resembles in principle the Quick prothrombin time test (p 57) but the preincubated mixture described above is substituted for the usual tissue (brain) thromboplastin.

The Oxford group and investigators from other laboratories have applied the thromboplastin generation test system to the study of very many problems among which we may list the following

- 1) testing of platelet functions in blood clotting. The platelet suspension may be tested (a) before and (b) after activation by plasma factors [225] (see pp 27-29) in normal subjects in thrombocytopenias [49] and in cases with functional platelet disorders (thrombasthenias or thrombocytopathias) [1445].
- 2) substances able to substitute for platelets in the thromboplastin generation test include (a) a weak tissue thromboplastin [38] (b) cephalin [354] (c) chylomicra suspensions [406].
- 3) diagnosis of hemophilia and related disorders [313-378]
 - (a) when the patient's $Al(OH)_3$ plasma gives a poor test the other reagents all being normal the result is presumptive evidence for hemophilia. Ordinarily AHF persists in fresh $Al(OH)_3$ plasma along with AcG (factor V) whereas PTC (Christmas factor) prothrombin proconvertin (factor VII) and probably factor X are removed by the adsorption.
 - (b) when the patient's aged (overnight) serum gives a poor test the other reagents being normal the result suggests PTC deficiency (Christmas disease). PTC ordinarily survives in the serum along with factor VII whereas AHF and factor V are gone as is prothrombin (consumed) and thrombin (removed by the natural antithrombin).
- 4) certain circulating anticoagulants interfere with the test (Hougie & Fearnley [227] Hougie [226]). The present author and colleagues [152] have recently proved that these inhibitors are specifically anti AHF and anti PTC respectively. We prefer our specific tests based upon the prothrombin consumption technique (p 46) because they make the distinction between the two inhibitors. The thromboplastin generation test is unable to do this. In other respects however it may be more sensitive than prothrombin consumption test methods. A careful comparative study of the two methods has not been made and there is as yet no true yardstick for evaluating the validity of quantitative data of these and also other (e.g. [282]) methods.
- 5) the question of intermediates;
 - (a) Biggs Douglas & Macfarlane [50] found evidence for participation of factor V (AcG) and factor?VII (proconvertin) in

used the two stage prothrombin method to assay residual prothrombin in serum after various intervals and thus determine 'prothrombin conversion rate'. This was much reduced in hemophilic plasma compared with normal. It could be brought to normal however by adding lung thromboplastin. In the careful preparation of the plasmas immediately centrifuging the citrated blood at low temperature the prothrombin conversion was found to be slower than in an earlier experiment. Conversely when hemophilic blood was kept in citrate for 24 hours before removing the plasma from the cells the subsequent test showed great improvement in the prothrombin conversion as well as in recalcification clotting times.

Eight years later with the advent of the silicone technique [251] Brinkhous [64] repeated efforts to obtain a platelet poor plasma and clearly showed that in normal plasma recalcification clotting times and 'prothrombin consumption' (or 'utilization') depended on the numbers of residual platelets. A platelet poor plasma became 'quasi hemophilic' in these respects but normal platelet poor plasma corrected the defect when added to the hemophilic. This was not so however when the hemophilic plasma was also 'platelet free'. He concluded in hemophilia there is a plasma factor required for platelet utilization.

In further pursuit of the prothrombin utilization problem Brinkhous and colleagues [281] compared data obtained by the two stage test with the Quick one stage prothrombin method in both dogs and human each being (1) normal (2) hemophilic (3) platelet poor (by centrifugation) (4) blood clotting in silicone. While both methods revealed the defective prothrombin consumption in (2) (3) and (4) the one stage values were not only higher but ran much above the original (0 time) value even in the normal. This would appear to be due to the evolution and persistence of the recently recognized serum factor (convertin see p 46) which accelerates thrombin formation. Evidently the two stage test is the more reliable measure of the prothrombin.

Quick [395] in 1947 also approached the prothrombin consumption problem via hemophilia and modified his one stage prothrombin test (see p 57) by addition of fibrinogen later substituting $\text{Ca}_3\text{P}_2\text{O}_8$ a sorbed plasma [386 387] in order to apply it to (1 hr) serum (cf Soulier [444]). Like Brinkhous Dr Quick and his colleagues showed deficient prothrombin utilization in hemophilia and in platelet poor (centrifuged) plasma [391]. They also studied the prothrombin consumption defect in (a) cases of thrombocytopenia [391 392] (b) a case of labile factor deficiency [390] and (c) after dicumarol [388]. In one thrombocytopenic splenectomy raised the platelet count and restored the prothrombin consumption [392 388]. Stefanini & Crosby [45-] have extended these observations.

f) Platelets and thromboplastin generation, intermediates. A new avenue of exploration of the blood clotting field particularly in relation to clinical bleeding disorders was opened up recently by the work of the Oxford University pathologists in the laboratory of Professor R C Macfarlane. In 1952 Dr Rosemary Biggs [47] observed the development of a plasma thromboplastin in the platelet rich recalcified plasma from a rare case of congenital prothrombin deficiency. Working

of Product I and the complete thromboplastin. The last named is moderately stable in simple preparations but loses activity in serum or when citrated or oxalated. The participation of factor V and factor VII is presumed in the formation of Product I and hence of complete thromboplastin but details of these relationships need further investigation.

(c) Hördler has studied the defect in thromboplastic generation in a case with an alleged specific inhibitor of factor V [223] and has data on the influence of factor V upon plasma thromboplastin and hence thrombin formation [224].

Discussion on intermediates. It appears to the present author that present methods are yielding conclusions which are suggestive rather than definitive. The reagents used in the cited experiments are crude materials for the most part and this raises some questions. In Macfarlane & Biggs [314] experiments for instance it was concluded that factors V and VII increased the potency of tissue thromboplastin. What if any is the evidence that the potencies of factor VII and factor V are not being increased? Serum contains much proconvertin as well as convertin and pre incubation with calcium and thromboplastin can activate the former to the latter and abolish the lag phase in the conversion of prothrombin (factor VII free) to thrombin [295]. Similarly a very minute trace of thrombin can activate proaccelerin to accelerin with similar abolition of the lag phase in purified thrombin forming systems [290]. Can the English workers state the complete absence of traces of thrombin in their test system? These are debatable matters and the important point is that the experimental test systems leave the results open to divergent interpretations. The final answers must await more definitive experiments. It would seem that the distinction between the thromboplastin forming and the thrombin forming reactions is not yet wholly established.

Conclusion. Despite a diversity of approaches and viewpoints modern investigators in this field are providing evidence of similar basic concepts and of a common goal of inquiry. It would now seem to be established that the many factors which participate in the thrombin forming reaction do so by a series of complex and inter related reactions in which the formation of certain intermediates would seem to be a logical necessity. Many but not all of the data can be interpreted in terms of thromboplastin generation.

g) Platelet factors related to clotting, etc. At an April 1955 Conference of the National Research Council's Coagulation Committee meeting in Washington D C W H Seegers [426 104] listed eleven experimentally demonstrable actions of platelets or platelet fractions in the blood clotting system. We quote his list verbatim.

- 1 Platelet factor 1 (Ac globulin like property)
- 2 Platelet factor 2 (Thrombin fibrinogen interaction)
- 3 Platelet factor 3 (Prothrombin activation with plasma co factor(s))
- 4 Platelet factor 4 (Antiheparin)
- 5 Clottable factor
- 6 Antifibrinolysin activity
- 7 Antithromboplastin activity
- 8 Clot retraction
- 9 Vasoconstriction (serotonin)
- 10 Snake venom factor
- 11 Ac globulin stabilization

thromboplastin generation from platelets Macfarlane & Biggs [314] also showed an increasing potency when an already active brain extract was preincubated with factors V and VII (cf [320 319 318]) This raises considerable question as to terminology and Macfarlane et al suggest that the term 'thromboplastin' should really apply to the product of the preliminary reactions which with calcium participates directly in the conversion of prothrombin to thrombin Since Howell (p 9) although not strictly in that author's sense the term thromboplastin has been widely used to designate any tissue extract used in activating prothrombin Brain thromboplastin for instance is the key reagent in the Quick prothrombin time test (p 57) Brinkhous and colleagues (p 46) distinguish between tissue thromboplastin as a 'complete' thromboplastin and cephalin or platelets as 'incomplete' or 'partial' thromboplastins They apply these ideas in their two stage prothrombin consumption test (p 46) and partial thromboplastin APTT assay [65 66]

Actually the historical term (p 8) for the postulated activator with calcium of the prothrombin conversion was 'thrombokinase' Hence there is much merit in Milstone's [339] suggestion to re define this term for the present purposes Milstone's work while confined to experimental test systems essentially anticipated much of the basic concepts advanced by the more recent English investigators Other American researchers notably F D Mann and associates [320 318] Flynn & Coon [165 166] have pursued the idea of intermediate complexes in the thrombin forming reactions although not emphasizing quite the same viewpoint as the above The distinction between thrombin generation [312 377] and thromboplastin generation is by no means clearly established at this time however (see p 49)

(b) Hougie [225] has studied the shortening of the recalcification time of platelet rich plasmas from cases with (1) circulating anti coagulant (2) hemophilia (3) Christmas disease (4) 'dindevan' (phenyl indanedione related in action to dicumarol and tromexan) and (5) normal bloods He concludes that there is another important intermediate at a later stage in these complex reactions The following summary of these ideas was kindly supplied by Dr Hougie in a personal communication According to Bergsagel & Hougie [40] APTT and PTT undergo a reaction with calcium to form an intermediate product (Product I) Product I reacts with platelets causing them first to agglutinate and then to lyse (Bergsagel) resulting in a sedimentable thromboplastin complex believed to be a complete thromboplastin This (a) differs from tissue (e g brain) thromboplastin (b) in that the one stage prothrombin time of stored and dindevan plasma considered to be deficient in both factor V (proaccelerin) and factor VII (proconvertin) respectively is normal with (a) but delayed with (b) This is true even when (a) is washed and re sedimented and used in high dilution The complete thromboplastin loses its activity on heating for 10 minutes at 56°C However after heating for 5 minutes to 100°C and re treating with Product I the activity is restored and may again be recovered in the (ultra)centrifugal sediment The heat stable component is thought to be cephalin and the heat labile component to be Product I Product I loses its activity after decalcification by Amberlite (ion exchange resin) and therefore calcium is believed to be an essential component

for the possibility of loss due to lability of the AcG like factor we inclined to the view that it might merely represent some of the plasma AcG adsorbed onto the platelets. This view is strongly supported in a recent study by Hjort et al [222]

7) A factor contributing to AcG instability was incidentally noted by Ware Fahey & Seegers [490]

8) A factor contributing to thrombin stability was incidentally noted by Travis & Ferguson [476]

9) A fibrinoplastic factor (the present author's term see p 17) aiding the thrombin fibrinogen reaction was claimed by Ware Fahey & Seegers [490] and also reported by van Creveld & Paulssen [481] and by Jurgens [261]. Travis & Ferguson [476] could not confirm this however. Platelet Factor 2 (Seegers) denotes this alleged activity

10) A clottable factor [490] refers to the clot like reaction of platelets to the addition of thrombin. The author's (unpublished) dark field observations indicate this to be a variant of the 'viscous metamorphosis' (p 35) of platelets and not any true clotting phenomenon

11) Platelet vasoconstrictor or serotonin has also been considered separately (p 39). Also ref Correll et al [94] and Humphrey & R Jacques [243]. Magalini & Stefanini [315] could not confirm Fenichel & Seegers' claim [121] for a clot retraction effect of serotonin

12) An antifibrinolytic factor was noted by Seegers' colleagues Johnson & Schneider [257] and confirmed by Stefanini [450]. Also see p 119

Conclusion This is obviously a rich field for further investigation to determine the exact identity of the factors involved and the way in which platelets can participate in the phenomena mentioned

h) Erkelens' thesis A recent comprehensive thesis [118] of the role of platelets in the coagulation process by A D Erkelens (Rotterdam) has appeared in Holland. Its chief conclusion is that plasma changes precede and are probably responsible for platelet breakdown. In platelet free plasma coagulation can be induced by kaolin and other particulate matter. As a result of the secondary structural changes of the thrombocytes after the formation of the first fibrin, a thermally stable thrombocyte factor is liberated stimulating the formation of thromboplastin. Therefore an accelerating and finishing function in the process of blood coagulation is assigned to the thrombocytes but no initiating function.

Stefanini [449] includes some detail as to platelet factors in his 1953 review. Ackroyd's recent review [1] cites "platelet factors 1 4 and these are so titled and discussed in much of the current work in this field. We shall not attempt any comprehensive review of all the literature on this topic but will present our own attempt to order the experimental facts in some logical order with a few selected references to each category

1) Platelet thromboplastin' (preformed) The best evidence for this is Chargaff's [78] preparation of 'platelet thromboplastin' as a lipoprotein centrifugate. From this he recovered 'platelet thromboplastic phospholipid' (? cephalin) in lipoidal extracts. That this is a weak and 'incomplete' thromboplastin is evident from the data of Quick [387]. Ware, Fahey & Seegers [490]. Travis & Ferguson [476] and others.

2) Platelet component (platelet Factor 3) in generation of plasma thromboplastin. Lenggenhager [287] used the term 'thrombokinin' and Quick [387] the term 'thromboplastinogenase' in looking at this from one point of view namely as if the platelet component were the activator of the plasma components (see AHF = 'cofactor I' and PTC = 'cofactor II' etc. p. 28). It is really semantic (or tautological) but most other workers prefer to present the problem from the opposite point of view namely as if the plasma cofactors activate the platelet precursor of a thromboplastin. Bergsagel and Hougie's views as to probable 'intermediates' in these reactions have been stated (p. 48). Alkjaersig, Abe & Seegers [12] have attempted to purify platelet factor 3 and guardedly state that it is unlikely to be a protein. In their best preparations it has an N/P ratio of 8/4/1. No discussion of the reactions which these authors claim for 'platelet factor 3' can be adequate without going into the many details of Seegers' current theories which we do not propose to review in the present dissertation.

3) Snake venom factor according to Lee, Johnson & Seegers [285] refers to some platelet factor which acting in conjunction with Russell's viper venom (itself a 'thromboplastin' with certain restrictions of potency [398, 436, 500]) is able to activate purified prothrombin rapidly in experimental systems. Interrelations between venoms and phospholipids is a topic with a considerable literature and we shall not explore this any further. See SUPPLEMENT.

4) Antiheparin action (platelet Factor 4) claimed by van Creveld and Paulssen [481] and recovered in a separate fraction by Deutsch et al. [104]. Other recent references [261, 490].

5) Antithromboplastic action: ref. Jurgens [261] thermolabile factor sedimentable in the ultracentrifuge.

6) Platelet accelerator (platelet Factor 1) was discovered by Ware, Fahey & Seegers [490] and probably accounted for some of the data of Quick [385] and of Mann, Hurn & Magath [320]. Travis & Ferguson [476] found only traces in well washed platelets. Allowing

PART II

EXPERIMENTAL

A GENERAL PURPOSE

The following experiments seek further knowledge concerning the role of lipids and platelets in the blood clotting mechanisms. Blood, its platelets, plasma and plasma fractions, and a number of tissue components are analyzed with regard to the various factors which they contain and which can be demonstrated to play a part in the interactions involved in these mechanisms. The significance of the platelets and some of the other factors is considered in relation to hemorrhagic disease. In vitro test systems are devised to demonstrate the modes of action of the various factors and agents studied. Many experiments are being performed by this means.

B GENERAL METHODS

The materials used in these investigations are for the most part derived from mammalian blood, namely dogs, humans, and beef cattle.

1 BLOOD COLLECTION

1a) Dog Blood samples of some 10-50 ml (or more) may often be obtained from the jugular vein of the unanesthetized dog with minimal discomfort using a syringe and needle technique, as in the case of humans (see 1b). For large amounts of blood it is desirable to sacrifice the animal under intraperitoneal sodium pentobarbital (nembutal) or similar anesthesia. The carotid artery is cleanly exposed and cannulated with a siliconed glass cannula or polyethylene tubing. Controlling the vessel with a soft rubber tipped bulldog clamp and avoiding tissue contact, e.g. by discarding the first few ml of blood, collection is made into anticoagulant, or otherwise, as needed, using glass (pyrex) Erlenmeyer flasks or siliconed bottles. Oxalation or citration requires 9 volumes of blood to 1 of 0.1 molar sodium oxalate or trisodium citrate. Sequestrene Na_2 (EDTA or 'Versene') is reserved for special collections, e.g. for platelet studies, the routine being to receive 5 ml of blood into a siliconed 1 oz. bottle containing 50 mg of the powdered chelating agent (cf. p. 276 of ref. [453]).

1b) Human An extensive testing routine [172] may be performed on 20-40 ml of blood obtained aseptically from the antecubital vein using a siliconed syringe with a sharp new short bevel No. 20 needle. Arquad 2-C (2 percent solution) coating of needles (p. 283 of ref. [453]) has little additional advantage. The blood is immediately transferred into anticoagulants, as in 1a, and 4-5 ml of blood is also clotted in a small (Wassermann type) tube held in the water bath at 37°C. This is

Other adsorbents used in some of our earlier work include $Mg(OH)_2$ [176 442] which is too alkaline in our opinion; type C^a $Al(OH)_3$ gel [381 52] $Ca_3(PO_4)_2$ [56 387]

4 FIBRINOGEN

For purposes of the experiments described in this thesis the most satisfactory fibrinogen is prepared from dog oxalated plasma which has been adsorbed with $BaSO_4$ as in 3. The fibrinogen is salted out at 1% sat $(NH_4)_2SO_4$ the centrifuged precipitate dissolved in saline and dialyzed against saline containing 0.005M trisodium citrate. Some purer fibrinogens will be described in certain experiments.

5 PROTHROMBIN PREPARATIONS

5a) Howell type [71] This was much used in our earlier studies before the advent of the modern types of preparation. It was made from Berkefeld filtered citrated dog plasma (see Experiment 22 p 91) by means of acetone precipitation (after heat defibrination) collecting the precipitate on filter papers washing with ether and drying. For details of preparation of solutions see p 91.

5b) Seegers type These are the purest prothrombin preparations to date. They are secured by adsorption of citrated plasma (bovine) with $Mg(OH)_2$ and subsequent elution with CO_2 under pressure see p 15 [435 431]. We have had some personal experience with this type of preparation but have used many that were kindly prepared for us by Dr Seegers ref [156].

5c) Eluate type In recent work including many of the experiments in this thesis we have obtained a prothrombin rich solution from dog human or bovine oxalated plasma by adsorbing with $BaSO_4$ (see 3 above) and subsequently eluting the twice washed (with distilled water) sediment with 1/3 volume 0.2M trisodium citrate see p 60. Analyses on these eluates will be described in several of the experiments.

C REAGENTS

1. COMMERCIALY AVAILABLE PRODUCTS

Chemicals such as various salts solvents etc. usually require choice of the best 'analytical' grade and careful and accurate preparation of solutions.

Special materials which are currently available include

1) Armour & Co.'s bovine fibrinogen plasma Fraction I¹ lyophile dried the preparative methods being based on the Harvard Plasma Fractionation Commission's cold ethanol technique [114]. We routinely remove traces of prothrombin etc. by $BaSO_4$ adsorption 1 g/100 ml of 1% solution of B.F. is sufficient.

2) Human fibrinogen prepared by this method and available for restricted distribution by the American National Red Cross.

3) Incubation mixture for performance of the two stage prothrombin assay (according to Ware & Seegers [495]) obtainable from the Difco Corporation (courtesy of Dr C. W. Christensen).

later centrifuged in order to recover the serum. As a routine for prothrombin consumption determination 0.5 ml of 0.1M sodium oxalate is added after exactly 1 hr immediately before centrifuging.

1b) Special blood donations, e.g. normal hemophilic PTC deficient etc. are secured through cooperation of the Blood Bank and the citrated or oxalated plasmas are reserved or fractionated for particular test purposes. Frozen storage at 20°C ('Deep Freeze') is a routine.

1c) Beef. Ox blood is obtainable at a cooperative slaughterhouse where it is possible to arrange for suitable large scale collections. With suspension of the cleansed animal and skill on the part of the butcher there is such a rapid flow of blood that very little objection can be raised to its slight contact with the wounded tissues. Several gallons of blood may be secured in thoroughly clean galvanized pails containing the appropriate amount of anticoagulant. The mixture is quickly transferred to large pyrex bottles kept chilled in buckets of ice for transport to the processing laboratory.

2 PREPARATION OF SERUM PLASMAS PLATELETS

2a) Natural serum. Whole blood is clotted in tubes (or bottles) held in the water bath at 37°C for 1 hr. The serum is then separated from the retracting clot by suitable centrifugation.

Oxalated serum. This is described in 1b) above.

2b) Plasma. e.g. oxalated or citrated are secured by centrifuging the blood containing the anticoagulant in glass, lusteroid or polyethylene tubes. The refrigerated (4°C) 'International' centrifuge is recommended and it is best to perform the operation in two stages namely 15 min at 1500 r.p.m. and recentrifugation of the supernatant cell contaminated plasma at 3000 r.p.m. for 1/2 - 1 hr.

2c) Platelet rich plasma. Versene treated blood p. 53 is transferred to siliconed tubes and centrifuged at 800 r.p.m. for 20 min. The supernatant plasma is rich in platelets and if only the upper portion is removed there will be a reduced contamination with leucocytes and erythrocytes.

2d) Platelet poor plasma. The top 2/3 only of the above (2c) platelet rich plasma is carefully removed and measured with a siliconed pipette. The measured volume is transferred directly into the 6 ml capacity siliconed tubes of the 'multispeed' attachment of the refrigerated 'International' centrifuge and spun for at least 45 minutes at 20 000 r.p.m. The clear supernatant avoiding the lowermost portion is all but free from platelets.

2e) Platelets. The sedimented platelets from 2d) are washed 6x with 0.85% NaCl (saline) containing 0.2% Triton (WR 1339 Rohm and Haas ref. [453]) and finally resuspended in 1/10 the volume of the original platelet rich plasma.

3 BaSO₄ ADSORPTION ETC. [98 463 410 194]

Ten minutes adsorption of plasma with pure BaSO₄ e.g. Merck's 'reagent' at 100 mg per ml removes prothrombin, proconvertin, PTC etc. leaving most of the fibrinogen, AcG, AHG etc.

b) The prothrombin time determination patterned after the original one stage test of Quick et al [393] is performed as follows To 0.1 ml plasma is added 0.2 ml of Ca tpin (equal volumes of 0.02M CaCl_2 and tissue thromboplastin e.g. 'Soluplastin' p. 56) and the appearance of fibrin timed with a stopwatch at 37°C

c) Thrombin forming factors, assayed by specific ONE STAGE methods By modifying the above technique we (and others) have overcome the non specificity of the original test and introduced specific bioassays for (1) prothrombin (2) (pro)accelerin and (3) (pro)convertin

The three methods differ in the choice of substrates (see below) They all follow the common procedure of adding 0.1 ml of 1:10 (or suitable) dil plasma (or serum or other agent) to 0.1 ml of the appropriate substrate in a 13 mm diam serological tube in a 37°C water-bath then adding 0.2 ml Ca tpin and timing the clot (film at edge) with a stopwatch

Ca tpin equal vols 0.02M CaCl_2 and Soluplastin (p. 56) or other suitable tissue thromboplastin A normal designated 100 per cent is the mean of a statistically significant number of assays on plasmas from healthy normal adult human subjects From (a) such a normal or better (b) a hyperreactive pool from a number of cases at or near the upper limit of normal variations a series of saline dilutions (preferred to other diluents) is made and the data are plotted as a standard reference curve Values up to about 120-130 percent of the statistical norm are included in the curve obtained by method (b) and most plasma unknowns will fall on this reference curve In the case of reagent testing variable dilutions are necessary

To overcome day to day variations of reagents and test conditions it is recommended that a 'control of the day' be selected from available personnel whose assay point on the standard curve has been repeatedly established This test will then orient the standard curve for the particular set of tests as shown in the following example

Patient 'X' gives a one stage clotting time (say for prothrombin) corresponding to 63 percent on the standard reference curve The day's control known to be 90 percent on the standard curve today gives a clotting time which reads at 81 percent Hence the corrected UNITAGE of the unknown 'X' is $63 \times (90/81) = 70$ expressed as percentage of the standard normal In the present study reciprocal prothrombin times are also expressed percentagewise in terms of a standard normal 'activity' Figures 9-10-11-12 show correlation graphs of four methods namely prothrombin time Method I (specific one stage prothrombin assay) Method II (orthodox two stage prothrombin assay) Method III (eluate assay method for prothrombin) They represent over 300 tests on plasmas from human cases both normal and with clinical variations to the limits of the test methods

The excellent correlation ($p = 0.95$) of prothrombin data obtained by the specific 1 stage (Method I) and the orthodox 2 stage (Method II see below) is graphically illustrated in Figure 10 Incidentally as shown in Figure 9 there is a very much poorer correlation ($p = .5$) of Method II and the unmodified prothrombin time test due to the now recognized fact that the Quick test is influenced by variables other than prothrombin All three specific methods show excellent correlation

d) Method I. Specific 1 stage prothrombin assay Our modification of the method of Owren & Aas [365] uses as substrate a BaSO_4

4) 'Simplastin' (Ca-thromboplastin) is available from the Warner Chilcott Co and is reliable for prothrombin time tests

5) 'Soluplastin' Schieffelin and Co (courtesy of Dr E W Blanchard) is the commercial thromboplastin used for most of the tests in the present study

6) Bovine thrombin Upjohn & Co (courtesy of Dr J T Correll) is preferred

7) 'Thrombin topical' (bovine) Parke Davis & Co (courtesy of Dr E A Sharp) also is good but is often unduly contaminated with fibrinolysin

Many individual investigators have provided us with samples of their materials which will be acknowledged in appropriate sections. Our own procedures and preparations will also be described and critically discussed in connection with their use in various experiments

2 TESTING OF REAGENTS

While the special qualitative and quantitative characterization of reagents will entail testings which are part of the individual experiments there are some general test procedures which are appropriately reviewed at this time. The following selected techniques represent the fruits of many years of experience. They embody experimental criteria based upon many lines of progress by a host of modern workers in this field. However many of them were not available in the earlier periods of our study when some factors much less techniques were still unknown. Hence these test methods are presented as recommendations in the light of which old experiments may be reviewed and re-evaluated and sometimes profitably repeated perhaps with modifications suggested by the newer ideas.

a) Fibrinogen assay (I) Gravimetric The common clinical routine [453-473] is quite useful for approximate determinations on isolated fibrinogen solutions. Add 0.5 ml bovine thrombin (500 units/ml) to 0.5 ml solution (e.g. plasma) diluted with 5 ml imidazole buffered saline. Imidazole buffer [331] of pH 7.3 is made by dissolving 1.72 g imidazole (Edcan Labs) in 90 ml 0.1N HCl and diluting with distilled water to 100 ml. One vol imid buff is added to 9 vols saline (0.85% NaCl). The fibrin clot is carefully and completely wound off on a tared glass rod washed with saline followed by distilled water and dried to constant weight i.e. overnight at 85°C and weighed.

(II) Colorimetric [348] Our [60] preferred method for highly accurate assays of purified fibrinogen is to recover the thrombin fibrin obtained as in (I) on a mounted dissecting needle washing digesting with a modified Mehl's biuret reagent then diluting and reading the color intensity with the Klett Summerson photoelectric colorimeter using No. 540 filter. The instrument is previously graduated against the above gravimetric (dry weight) and macro Kjeldahl (fibrin N) determinations.

(III) Turbidimetric In 1942 the author [135] used the Evelyn photoelectric colorimeter with No. 540 filter as a turbidimeter. Supplementary to other studies it was noted that the turbidity of clots formed by adding thrombin to a 1:10 dil plasma could accurately measure the addition of fibrinogen in amounts as small as 7.5 mg/100 ml. A recent micro method [512] is based on these principles.

f) Specific 1 stage proconvertin assay uses as substrate bovine plasma twice filtered through special '20" asbesto (Seitz type) pads obtained from the St Gallen Co Switzerland according to the original recommendations of Owren & Aas [365]. This filtration is designed to remove nearly all the proconvertin but to leave a sufficient amount of prothrombin. Our tests usually reveal about 60% of the original prothrombin and a control prothrombin time test (with 0.1 ml saline instead of test plasma) of 120 sec or longer. A new recommendation [2] to employ a wood charcoal 'L 50' (Springfield Facing Co Williamsett Mass) may be noted. We have at times supplemented our substrate with proconvertin free bovine prothrombin prepared by the method described for human plasma fractionation [295]. Fibrinogen and other factors are satisfactory in this substrate but like the prothrombin substrate (p 58) it may lose AcG and require supplementation of this in the test plasma dilution.

g) Method II. The orthodox TWO STAGE prothrombin assay. The original two stage assay [496] has been modified [495]. Ferguson et al [153] have recently made a critical restudy of the two stage prothrombin assay method both as to technical considerations and in application to the investigation of bleeding disorders. Our improved test adds 1" bovine proconvertin (see above) as well as AcG (the 'modified' method). This is actually essential only in very rare instances when testing plasmas etc which are extremely low in proconvertin e.g. some congenital hypoproconvertinemics. Briefly detailed the method requires four steps namely:

Step 1. Fibrinogen 0.5 ml plasma + 0.4 ml saline + 0.1 ml thrombin (bovine Upjohn's 20u/ml) remove clot with glass rod over 15 minutes

Step 2. Dilution Appropriate (e.g. 1:30) dilution of 0.1 ml (1) with 1:100 (saline dil.) AcG + proconvertin

Step 3. Activation 0.6 ml (2) + 1.8 ml incubation mix consisting of 11.5 saline, 1 imidazole buffer, 4 purified (Ca free) acacia 15" (Willis Corp), 0.6 Soluplastin (Schieffelin & Co), 0.9 CaCl_2 (0.1M)

Step 4. Clot timing 0.4 ml samples of (3) removed at 1 min intervals are tested with 0.1 ml fibrinogen at 28°C (25°-30°) noting the minimum reached. This should be in the 13-17 sec range.

Our best standard fibrinogen is simply prepared from oxalated dog plasma BaSO_4 adsorbed as usual (see p 55) precipitated at 1/4 saturation $(\text{NH}_4)_2\text{SO}_4$ redissolved in 1/3 orig vol saline and dialyzed against citrated saline (0.005M sodium citrate). Frozen stored (20°C) in 10 ml lots it keeps well for many months with a remarkably constant reactivity (see p 62). Occasionally a small amount of sediment appears on first thawing but this can be centrifuged off and the bulk of solution remains clear thereafter even through repeated freezings and thawings.

Computation of unitage E.g. if a 30x dil is used in stage (2) and the minimal clotting time (reached say in 3 or 4 min of step (3) incubation) is 14 sec and hence the correction factor from Ware & Seegers' tables [495:256] is 1.1, $1.1 \times 5/4 \times 4/1 \times 30/1 \times 2/1$ (i.e. respective dilutions at steps 4, 3, 2, 1) = 330 units/ml

h) Method III. BaSO_4 citrate ELUATE technique a new two stage method developed in the author's laboratory. METHOD 3 ml

adsorbed beef plasma fortified with 1/10 vol of a bovine serum proconvertin preparation. This proconvertin is prepared from ox serum by the same method essentially as we [295] used in the case of human plasma. The serum was obtained by recalcifying oxalated beef plasma and centrifuging after standing for 18 hrs at 4°C. Interestingly this reagent although prepared from serum is mainly unchanged proconvertin. We deduce this by observing that it does not abolish the 'lag phase' in prothrombin conversion as compared with convertin (proconvertin pre incubated with CaCl_2 and thromboplastin) which does.

The prothrombin substrate is designed to contain the normal plasma amounts of fibrinogen, proaccelerin and other possible factors not adsorbed on the BaSO_4 . The BaSO_4 has removed the prothrombin (hence its suitability as a test substrate), the proconvertin (hence the re addition of this factor), PTC and possibly other materials which are not believed to play any role in the conversion of prothrombin to thrombin in the presence of Ca^{++} . Frozen stored in 5 ml lots at 20°C this prothrombin substrate remains serviceable for many months. Occasionally it loses significant amounts of AcG although this is far more stable in bovine than in human plasma. In such case the substrate should be assayed for prothrombin by the two stage method and if this is satisfactory the substrate can be restored to usefulness merely by using a suitable dilution of AcG instead of saline in the (1/10) dilution of the test 'unknown'.

Serum AcG (accelerin) as used for this and other (see two stage etc.) purposes is prepared according to the directions of Ware & Seegers [495] i.e. ox serum aged several hours is adsorbed with 4g/100 ml BaCO_3 . BaSO_4 can be substituted for the carbonate.

c) Specific 1 stage proaccelerin assay uses as substrate 'aged' human oxalate plasma as originally recommended by Quick & Stefani [395]. For some time (e.g. [297]) we recommended BaSO_4 adsorption (the usual 100 mg/ml) of the unknown plasma before the routine 1/10 dilution prior to adding to substrate etc. However any additional prothrombin which would be carried over in the diluted unknown to the normally prothrombin rich substrate can be shown to have a negligible effect particularly when the reference standards are handled in the same way. The easiest method therefore is to employ untreated 1/10 'unknown' plasma etc. as in the other specific 1-stage methods. In reagent (e.g. AcC) assays there is again no need for pre-treatment unless the material contains a large amount of prothrombin.

'Aging' of the substrate plasma is best accomplished by freezing and thawing (which always reduces the labile proaccelerin) and storage in the ice box at 4°C for one or two weeks (or longer) until the regular prothrombin time test (0.1 ml plasma + 0.2 ml Ca^{++}) at 37°C gives clotting times in excess of 60 seconds.

Aged plasma should contain normal amounts of fibrinogen, prothrombin, proconvertin etc. Frozen stored it is serviceable for many months and if a small amount of sediment appears on thawing this can be removed by centrifugation and the residual supernatant found to be little affected in most instances. A very fresh normal plasma must be used for the standard of reference and for the daily control. We have found it best to use a single well known 'normal' for both

Standards for the accelerator factor assays are a series of activation curves obtained with several dilutions of a comparable normal whether (k_1) plasma or (k_2) platelet suspensions after the manner of the Ware & Seegers two stage AcG assay [493]

1) Thromboplastic factor assay by the eluate method: Using essentially the same procedure but substituting for the regular thromboplastin ('Soluplastin') any material containing a thromboplastic factor can be assayed e.g. tissue thromboplastins (ℓ_1)

2) Platelet thromboplastic factor is routinely assayed in our laboratories by this technique. It involves platelet preparation (as above) and normal platelet standards with the reference series of dilution curves (activation curves)

Cephalin and a variety of lipids and tissue thromboplastic extracts will be studied by this method in experiments to follow

D INHIBITOR MECHANISMS

These will barely be mentioned in the present thesis but it must be noted that certain inhibitors of the thromboplastic mechanisms or perhaps of other components in the prothrombin conversion reaction can be demonstrated by delay in thrombin formation (compared with inhibitor free controls) in such test systems as these of the eluate method. Other prothrombin activation tests (showing inhibitory phenomena) will also be used in the present investigation

1 Heparin (with its cofactor) is an important experimental addition in some of the present test systems

Testing for heparin in blood or plasma is usually done by (a) whole blood clotting time tests or (b) protamine titration (with which we prefer to use a plasma recalcification system) but details of these techniques will be omitted

2 Antithrombin tests

a Plasma thrombin clotting time test The clotting time (at 37°C) of a test plasma mixed with a standard solution of thrombin and compared with a similar experiment using a normal plasma is often suggestive evidence of some 'immediate' (or rapidly acting) antithrombic factor. An example is seen in the new born infant [172]

b Serum antithrombin refers to the 'progressive' loss of thrombic activity when a standard thrombin is incubated with serum (or other fibrinogen free material) and retested with fibrinogen at successive time intervals. Saline may be substituted in the control

This test is performed with many of our test materials when we wish to demonstrate the presence or absence of antithrombin. An example is seen in Table VII

Often however it is enough merely to retest a thrombin forming mixture at intervals to observe definite antithrombic action. This is always seen for instance in the orthodox 2 stage prothrombin test and raises some theoretical questions as to the true end point. In practice at the high dilutions tested this is usually ignored however

E STUDIES ON ARTIFICIAL CLOTTING SYSTEMS

1 TEST METHODS In general test systems are quantitative using

oxalated (not citrated) plasma is adsorbed 10 min with 300 mg BaSO₄ (Merck's) and the sediment recovered by centrifugation is washed with distilled water and then eluted for 10 min with 1 ml of 0.2M sodium citrate. The eluate recovered by centrifugation contains essentially all the prothrombin proconvertin and PTC of the original plasma. It lacks fibrinogen proaccelerin (except for traces) AHF and most of the plasma inhibitors (e.g. antithrombin) usually.

1) Eluate assay (Method III) for prothrombin, by new two stage, 0.1 ml eluate is incubated at 28°C (or approximate room temperature) with the following activator system: 3.6 ml saline (0.85% NaCl), 0.5 ml (i.e. 1/10 vol) imidazole buffer (pH 7.3), 0.1 ml bovine proconvertin (see p. 58), 0.1 ml AcG (BaCO₃ bovine serum, see p. 58) diluted 1:8, 0.1 ml 'Soluplastin' (Schieffelin & Co's thromboplastin) and finally 0.5 ml 0.15M CaCl₂. At 1 min intervals 0.2 ml samples are tested with 0.2 ml fibrinogen (Armour's bovine 'Fraction I' 1% treated with 1 g BaSO₄/100 ml). Clotting times recorded with a stopwatch usually reach a minimum within 5 minutes and because of lack of inhibitors are typically stable or nearly so for many minutes thereafter.

Minimum clotting times are converted into prothrombin 'percent age' of normal by reference to a standard curve of data obtained from dilutions of a normal pool of plasmas as described under the 1 stage assays. A 'standard of the day' is also routinely used in the manner previously outlined (p. 57). Illustration of the technique used to assay standard eluates is seen in Experiment 10 (p. 75).

Correlation between Method III and the orthodox 2 stage (Method II) prothrombin assays for the same large group of (300) human cases as studied in the other comparisons of prothrombin methods is shown to be very satisfactory in Figure 12.

j) A 'proconvertin index' by modification of the eluate method: By omitting the 0.1 ml of proconvertin from the activator system in the previous test and securing the first minute test without it, a clotting time value is obtained which using the reference curve indicates the initial velocity of the prothrombin activation. This is due chiefly to the proconvertin present in the plasma eluate. By then adding the 0.1 ml proconvertin and continuing the usual incubation and testing the prothrombin activation is carried to completion. The ratio of percent age units in the 1 min test divided by the final (e.g. 5 min) percentage is a 'proconvertin index' which we have found to give a fair degree of correlation with the routine 1 stage specific proconvertin assay (p. 59).

k) 1) Eluate method of assaying (1) accelerator or (2) thromboplastin. A STANDARD eluate is made essentially by the method described for human plasma but using bulk volumes of either (a) dog or (b) bovine oxalated plasma. Using this as a substrate the prothrombin conversion may be studied at 1 min or other suitable intervals with the regular activator system modified by substitution for the cited (1) accelerator globulin or (2) Soluplastin of any other accelerator or thromboplastic material it is desired to study.

k₁ k₂ The new two stage AcG assay can be used for example on (k₁) fresh plasma (BaSO₄ adsorption is necessary) or (k₂) platelet suspensions (fresh) to assay their 'accelerator' property. See p. 54 for method of preparing washed platelets.

in this way that the National Institutes of Health standard is maintained

c) Standardization of fibrinogen. It is reasonable to standardize every batch of fibrinogen by repeated clotting tests with a standard thrombin as performed at N I H. In our laboratories this is not attempted. Either the particular batch of fibrinogen unstandardized is regarded as the 'standard' for a particular set of experiments relative c t values being acceptable in the absence of any evidence of fluctuations. Or when a particular fibrinogen is used for routine two stage prothrombin assays of plasma from normal subjects and human clinical cases sufficient experience is secured with the fibrinogen to characterize its reactivity using a statistical mean of cases with normal plasma prothrombin unitage [153]. We have prepared many dog fibrinogen preparations (p. 55) with which the two stage prothrombin assay gives close to 300 units/ml human plasma which is a very acceptable value.

d) Clotting times and thrombin concentration. Returning to the first method while this has no value in terms of any absolute unitage it nevertheless gives perfectly valid relative values if some reference series of thrombin dilution is used under the same experimental conditions. The fundamental principle is that the shorter the clotting time under standard conditions the more the thrombin activity or relative concentration. This is here put into practical use being validated as mentioned only for a given set of comparative experiments. By experimentally obtaining the data and plotting graphically as a thrombin dilution curve (e.g. Figure 5) the observed clotting times in any variation of the experiment may be read off as a 'percentage of the original (strongest) thrombin'. Much valuable information does not even require exact quantitation in this way. For instance a very long clotting time is clearly a matter of a very low thrombin concentration and two such tests may be far below the quantitative limits of the method and yet be significantly different by simple inspection of the data.

4 THE INVERSE LAW

In the example given in Figure 5 (see p. 18) quite by chance a very fair approximation to the 'inverse law' [141 162 20] was obtained. In our experience the true variables responsible for the usual divergence of data from this 'law' have not been defined and we do not usually attempt to secure this exact type of linear reference curve. A recent (unpublished) study of one possible variable however is illustrated in the following experiment.

F EXPERIMENTS

1 EXPERIMENTAL CONDITIONS MODIFYING THE THROMBIN FIBRINOGEN REACTION

The thrombin fibrinogen reaction has been discussed on pp. 16 et seq. in the introductory part of this thesis. Among the data there reviewed are those of Boyles, Ferguson & Muehlke [60] dealing with a number of experimental variables in the thrombin fibrinogen reaction. The first two experiments of the present thesis add new data obtained

accurate measurement of amounts of reagents including use of volumetric (e.g. serological) pipettes and other glassware always kept scrupulously clean with a cleansing routine involving detergent (Dural H) sulfuric acid chromate solution distilled water and drying oven. Timing of many procedures is facilitated by electric chronometers and mechanical stopwatches. Clotting times are used as 'end points' in the great majority of experiments.

2 CLOTTING TIME as discussed on p. 18 is an empirical observational datum which becomes significant only under strict conditions which will be defined for the individual experiments.

3 THE THROMBIN FIBRINOGEN REACTION

The key reaction in the physiological mechanism of blood clotting is the conversion of fibrinogen into fibrin through the agency of thrombin. The first step in our investigation therefore must be an evaluation of the time relationships of the thrombin fibrinogen reaction.

a) Fibrinogen and clotting time Fibrinogen concentration over a considerable range [60] has remarkably little effect on clotting time (c.t.). Of much more significance is the reactivity of the particular fibrinogen solution meaning the particular clotting time it will show with a given thrombin preparation under the defined test conditions. There is no real information to date upon which we can predict the reactivity of a fibrinogen. It is also not exactly known what determines the potency of a particular thrombin preparation. However any of the modern highly purified and stable thrombin preparations may be relied upon to give a definite clotting time reproducible within a small limit of experimental error in many repetitive tests conducted with a given good fibrinogen solution. Under carefully controlled and standardized experimental conditions repeatedly using the same substrate fibrinogen comparative clotting times afford a valid though relative measure of thrombin potencies. Actually two variants of method have both been widely used. In the first the thrombin concentration is varied and the respective clotting times used as a measure of relative thrombin potency. An example is given in Figure 5 in the introductory section. In the 'method II' (p. 59) the thrombin material is diluted to give a standard clotting time and relative dilutions are used as the measure of thrombin activity.

b) Standardization of thrombin The National Institutes of Health (Bethesda, Maryland) provides a thrombin standardized by this second method the original (revised) procedure being that of Smith, Warner & Brinkhous [443] as modified by Ware & Seegers [495]. The method was originally (1934) developed to assay prothrombin in terms of the thrombin unitage developed after maximal activation to thrombin. The unit whether for thrombin or activated prothrombin is defined as that potency of the product which gives a 15 second clotting time of a particular standard fibrinogen under the stipulated experimental conditions. Difficulties in securing a standard fibrinogen are insuperable as previously mentioned but a practical solution has been to have many testings of a particular lot of thrombin and then to retest many times when the original fibrinogen needs replacement with a new batch. It is

also have been removed in the isoelectric method of purification. Arguing for only a trace impurity is the observation that the $(\text{NH}_4)_2\text{SO}_4$ purification of Armour's fibrinogen gave a 97% clottable preparation which failed to obey the inverse law in the experiments of Boyles and the author [60]. Ammonium sulfate fractionation does not eliminate the Lorand factor [439].

Experiment 2 (1956) Table III

Purpose In the light of the cited recent work [307-439] clearly relating the Lorand 'serum factor' to calcium and the opacity, rigidity and urea insolubility of fibrin clots, is it possible to explain the anomalous action of calcium salts in a limited concentration range, namely shortening the clotting times of thrombin-fibrinogen mixtures on the basis of the reactions involving the serum factor?

Test Under constant volume and fixed thrombin and fibrinogen (I, II) concentrations, CaCl_2 was added to the final molarity noted and clotting times determined as in the preceding experiment.

Results are given in Table III. They show the usual (p. 19) accelerating effect of certain small concentrations of calcium ions.

Conclusion The usual acceleration of clotting in a certain range of calcium salt concentrations, here 0.001 to 0.01 molar, is evident. There is a definite optimum at 0.005M with all three fibrinogens. It must be concluded therefore that the Lorand serum factor is not significantly concerned with these effects of calcium ions on thrombin-fibrinogen clotting times.

2. EXPERIMENTAL EVIDENCE THAT CALCIUM IS NOT AN ESSENTIAL COMPONENT OF THE THROMBIN-FIBRINOGEN REACTION

Since the careful work of Hammarsten [206] (see p. 7) it has been reasonably established that calcium salts, i.e. Ca^{++} ions, are not essential to the thrombin clotting of fibrinogen. Howell's comment [238] that there might still be room for doubt, however, indicated the need for final proof that the trace of (protein-bound) calcium in the clotting reagents (fibrinogen as well as thrombin) is without specific influence on the clotting reaction.

Experiment(s) 3 (1937-56) (cf. Table XX)

Purpose To study the clotting of a completely decalcified thrombin-fibrinogen system.

Methods (A) In a 1937 experiment [128] the author prepared thrombin from Howell type prothrombin (p. 90) by maximal activation ('ripe' after several hours) with calcium and cephalin. In three experiments this thrombin was electrodialyzed for 2 hrs, 8 hrs, 13 hrs, respectively, in a Bradfield 3-compartment electrodialyzer.

by the author recently which deal with certain aspects of the serum (fibrin) factor problem (cf pp 20 21)

Experiment I (1956) Table II Figure 13

Purpose To investigate possible relationships of thrombin fibrinogen clotting times to the 'serum (fibrin) factor' of Laki & Lorand

Reagents and Methods

Preparation of 'serum factor' free fibrinogen (ref Laki & Lorand [277]; Lorand [304 305 307] Shulman [439] Stock fibrinogen: 1% Armour's bovine (plasma) Fraction I adsorbed with BaSO_4 to remove traces of prothrombin etc (p 55)

Fibrinogen I (Control): Stock fibrinogen dialyzed 2 hrs against 2M sodium chloride followed by overnight dialysis against 0.85% NaCl (room temperature) Thrombin clots of this solution remained insoluble in equal volume of 3M urea whether NaCl or CaCl_2 was added

Fibrinogen II Stock fibrinogen dialyzed 2 hrs against 2M sodium bromide followed by overnight dialysis against 0.85% NaCl The bromide treatment removed much of the serum factor but the test clots were still only partly soluble in urea in the presence of NaCl and negligibly soluble in the presence of CaCl_2

Fibrinogen III Fibrinogen II a week later was diluted 20x with distilled water and isoelectrically precipitated with 0.1M acetic acid with maximal turbidity at pH 5.5 The precipitate was recovered by 30 min centrifugation at 350 r.p.m. and redissolved in 0.85% NaCl to original volume Thrombin clots were now nearly all soluble in 1.5M urea even with added calcium only a trace remaining after 24 hours

Testing fibrin clots for solubility in urea 0.2 ml Fibrinogen + 0.05 ml 0.1M CaCl_2 (or 0.85% NaCl) + 0.05 ml thrombin (bovine Upjohn's 10 units/ml) treated 10 min later with 0.3 ml 3M urea

Clotting time tests with the above three fibrinogens Clotting times are measured with a stopwatch at constant temperature (25°C) on adding to 0.2 ml of the respective fibrinogens 0.2 ml of bovine (Upjohn's p 56) thrombin in siliconed tubes which are used in order to avoid any loss of thrombin by adsorption onto glass The thrombin was actually added at twice the strength of the final concentration stated in Table II

Results are shown in Table II and in Figure 13 In the graphic presentation in the cited figure relative thrombin concentrations are plotted against the reciprocal of the clotting times (1/c t)

Conclusion There appears to be a much better linearity in III and some improvement in II as compared with I (Figure 13) suggesting that the 'serum factor' of Lorand may indeed be a variable in causing deviations from the 'inverse law' It is not known how valid the criterion of urea solubility of recalcified clots is in determining freedom from the Lorand factor It is possible therefore that further progress may be made along the present lines There is an unexplored possibility that disturbing factors other than the Lorand factor may

silicone Series I plasmas were kept in silicone but tested on both types of surface. Series II were (G+) treated with powdered glass for 1 hr at 34°C in order to obtain a large measure of action of wettable surface. The control series II (S) were held in silicone for the same period at the same (34°C) temperature. Besides the Ca the following additives were tested: (1) saline (2) intact platelets (carefully preserved in silicone) (3) platelet extract (frozen thawed and triturated with saline) (4) brain cephalin. In another series of experiments simply conducted in glass (pyrex tubes) (1) was repeated and tests were also made with (5) tissue thromboplastin (Soluplastin) (6) crystalline trypsin (100y) (7) Loomis' [302] bovine fibrinolysin (4 mg). The data are shown in Tables IV and V.

Results Table IV: In (1) with Ca and saline only no clots were obtained in 24-48 hrs whether in glass or in silicone. This unequivocal result is difficult to achieve and was repeated on only two or three occasions. In many more similar experiments clotting did occur in glass in from one to many hours and longer in silicone.

In (2) and (3) clotting was restored with insignificant differences due to the manner of treating the platelets. There was however a consistent and significant difference between the tests on plasma only in contact with silicone and those exposed either (G) immediately or (G+) over a period to wettable glass surfaces. The glass clotting times (170-210) may be compared with the silicone clotting times (435-660). The somewhat longer c.t.'s in the 1 hr experiments probably mean some loss of labile factor (AcG). That the plasma is actually altered by the surface contact is shown by the finding of the shorter clotting times also in the tests in silicone (II S) after transfer to such tubes of the (G+) glass incubated samples.

In (4) cephalin is shown to act in much the same way as platelets. Actually it is even more effective in the concentration studied. The accelerator effect of glass on some component of the platelet free plasma is also evident in the cephalin experiments.

In the Table V data (1) the non clotting control is repeated (5) tissue thromboplastin is observed to give the very rapid clotting time of 7 sec exactly corresponding in fact to the typical prothrombin time (Quick test) on ordinary dog plasma; (6) trypsin and (7) fibrinolysin are also effective. That the two proteolytic enzymes of these last tests show similar 'thromboplastic' properties is an interesting observation which will require further investigation (see later).

Discussion None of the earlier efforts to establish the essential role of platelets in normal blood clotting were sufficiently convincing. Fuchs [178] in 1930 claimed the obtaining of a spontaneously incoagulable human plasma by high speed centrifugation in paraffined glass ware but Feissly (1934) and Smith Warner & Brinkhous (cited [64]) were unable to confirm this work. Introducing the silicone technique in 1946 Jacques Fidler, Feldsted & MacDonald [251] noted the prolongation of natural and recalcification clotting times as platelet counts were more and more reduced by increasing centrifugation in siliconized tubes. However they were unable to get rid of all platelets and did not obtain an incoagulable plasma. In the following year Brinkhous

with collodion membranes. The 110 volt direct current reached a maximum amperage of 0.3 to 0.4 amps in $\frac{1}{4}$ hr and fell to 1 or 2 milli amps in about 2 hrs. Distilled water was run continuously through both electrode (end-) chambers. The thrombin began to flocculate out at the anode membrane of the middle chamber in about 15 min and to sediment. Later it underwent partial migration to the cathode membrane. The diphasic behavior was explained by a change in pH of the unbuffered material over to the acid side of the isoelectric point of the globulins present. The final precipitate was recovered by centrifugation and redissolved in dilute Ca free KOH to pH 7.5 (phenol red indicator). The water clear supernatant always showed some relatively weak thrombic activity. Neither the redissolved precipitate nor the supernatant showed the least trace of turbidity on adding test reagent pot. oxalate and spectroscopic tests for traces of Ca were negative. The test fibrinogen was obtained from Berkefeld filtered (see p. 69) citrated dog plasma 'deprothrombinized' by $Mg(OH)_2$ adsorption [176] and precipitated (x3) at 1% sat $(NH_4)_2 SO_4$ reprecipitating at $\frac{1}{2}$ sat NaCl and redissolving in dist. water. We were unable to electro dialyze the fibrinogen without denaturation but relied upon excess of oxalate or citrate to decalcify it as indicated by a negative spectroscopic test for calcium. The most crucial test was performed after 18 hrs. incubation of both (a) the 13 hr. electro dialyzed thrombin and (b) the purified fibrinogen with equal volumes of (1) N/1 pot. oxalate (2) N/1 sod. citrate. On mixture of 1 ml amounts of thrombin and fibrinogen thus treated and still in the presence of the strong inhibitory and decalcifying salt(s) firm clots were obtained in about $\frac{1}{2}$ hr.

An interesting incidental observation was clot lysis after several hours indicating activation of the fibrinolytic enzyme system contaminating the thrombin preparation.

(B) Decalcification of proteins has subsequently become very simple with the advent of the ion exchange resins (p. 275 of Stefanini & Damehek [453]). We have repeatedly subjected both thrombin and fibrinogen to the cationic exchange resins Amberlite and Dowex '50' (sodium treated) and observed perfectly normal clotting of the subsequent mixtures (see Table XX).

Conclusion It is therefore concluded that protein bound calcium while contributing (like sodium potassium etc.) to the charge patterns on the protein molecules is not essential for the thrombin fibrinogen reaction and that thrombin in no true sense is a calcium compound.

3 EXPERIMENTS ON THE CLOTTING OF PLATELET FREE PLASMA

Experiment 4 (1949) [147] Tables IV, V

Purpose To study the clotting properties of platelet free plasma

Method Citrated dog plasma was obtained with the siliconed technique after 16 hr. centrifugation at 20,000 r.p.m. in the refrigerated 'Multispeed'. Equal vols. of each plasma sample were tested at 38°C for clotting times with 0.025M $CaCl_2$ (G) in glass and (S) in

preparing from it. It must be reported that the advances to date while encouraging still leave much to be desired. Nevertheless this should turn out to be a promising line of investigation leading toward the goal of purified individual blood clotting factors for specific therapeutic use in human deficiency cases.

4. EXPERIMENTS WITH THE BERKEFELD (BACTERIOLOGICAL) FILTER TECHNIQUE (cf. Experiment 2)

In 1913 Cramer & Pringle [95] claimed that the passage of citrated plasma through a Berkefeld filter rendered it incoagulable on subsequent recalcification but restored to clotting again on the addition of platelets. They concluded that they had (a) discovered a simple technique for 'deplateletizing' plasma and (b) proved that platelets were essential for normal clotting.

W. H. Howell at once set one of his pupils, C. H. Goddard (1914) [192] to repeating this experiment. On carefully perusing the English data Goddard noted that some of their Berkefeld filtered plasmas did clot on simple recalcification although delayed perhaps for several hours. One dog that had been fed three hours prior to bleeding gave a filtrate which clotted readily in 5 min. i.e. practically a normal recalcification clotting time. Goddard performed many Berkefeld filtrations and divided the filtrates into (1) first portion (2) intermediate and (3) last portion. It was found that only the first portion which was slow in appearing through the fine pores of the diatomaceous filter showed marked clotting defects. In fact it was often incoagulable even with thrombin, proving that most of the clotting factors even the fibrinogen had been removed. The last portions always clotted readily on simple recalcification. The intermediate portions varied and not uncommonly would clot poorly if at all on adding calcium but quite well if platelets, brain extracts or cephalin were also added. Prothrombin could be recovered by Howell's acetone method even from some of the intermediate filtrates which did not clot satisfactorily with Ca alone.

For a number of years the present author [126-130] routinely employed the Berkefeld filtration of plasma prior to preparing Howell type prothrombin. By the simple bacteriological laboratory trick of inverting a large test tube over the candle the filtration was invariably rapid and satisfactory. Test results confirmed Goddard's in the finding of good clotting on simple recalcification. Clotting times might be as long as 10-12 min. instead of the usual (unfiltered) plasma's 2-3 min. but could easily be restored to normal or better by addition of a small amount of cephalin [86].

Conclusions. The previously cited more recent work with the still cone technique and high speed centrifugation undoubtedly proves that the present author's views of an earlier period were erroneous in believing that all platelet material could be removed by Berkefeld filtration. We were looking for some plasma thromboplastin of other than platelet origin and well remember Howell's caution (p. 480 of ref. [233]) that platelets may very well disintegrate when roughly handled in ordinary centrifugation and in Berkefeld filtration. There were important errors in Howell's other views however.

[64] did succeed in obtaining a platelet free human citrated plasma with the silicone technique refrigeration and repeated centrifugations over a total period of 1350 min using speeds up to 14 000 r p m. The reported final preparation recalcified in glass at 28°C did not clot in 30 hrs. Prothrombin conversion was delayed in these 'quasi hemophilic' plasmas but prothrombin assays were normal. 'Thrombin topical' and 'thromboplastic extract from beef lung' clotted them as rapidly as normal whole blood. Addition of platelet suspensions reduced the clotting time to 6-9 min. They destroyed added thrombin normally. The platelet free normal plasma was able to correct the clotting defect of hemophilic whole blood but not of recalcified platelet free hemophilic plasma. Correction did occur in the plasma mixtures however if some platelets whether normal or hemophilic were provided. Hartmann & Conley [209] after many failures finally succeeded in obtaining a platelet free incoagulable canine plasma.

Patton Ware & Seegers [369] used a special technique in collecting blood without anticoagulant from a dog for siliconed tube centrifugation in the refrigerated 'Multispeed' at 22 000-23 000 r p m for 5 min-5 hrs. Some of the native plasmas thus obtained remained fluid for longer than 72 hrs at room temperature. There would usually however be some fibrin formed whether in glass or in silicone 'with about equal frequency'. Prothrombin analyses immediately after the centrifugations showed normal values compared with an oxalated plasma sample obtained at the same time as the special blood collection. The more highly centrifuged samples retained the same assay levels of prothrombin for several hours. Special (indirect) tests however showed the formation of traces of thrombin. The fibrin noted represented only a small portion of the fibrinogen.

Conclusions Our experiments [147] therefore confirm the few previous workers' evidence pointing to the fact that platelets are normally essential for blood clotting. The data on 'surface' effects causing some alteration of the platelet free plasma are original as is our use of cephalin and the enzymes in this type of experiment. The fact that a lipoid (cephalin) and the other cited reagents can replace platelets in what we believe to be a 'thromboplastic' type of action (see later) opens up some very intriguing questions.

Addendum Owing to the technical difficulty in obtaining large quantities of completely platelet free plasma the author (and others) have not progressed to the logical procedure namely to use such material as the starting point for the attempted preparation of plasma clotting factors free from all traces of natural 'thromboplastin' (at least of platelet origin). Mere 'platelet poor plasma' is insufficient for satisfying this rigid criterion although the greater the success in this direction the better would seem to be claims for purity in preparing prothrombin, proconvertin, proaccelerin, antihemophilic globulin, PTC and other factors. As a member of the Coagulation Committee of the Harvard Plasma Fractionation Commission the author has followed the recent use of the ADL Cohn Fractionator in the preparation of deplateletized human plasma [460] and has tested some of the fractions (e.g. prothrombin, accelerator globulin, etc.) which Dr. Surgenor is

room temperature with an equal volume of benzene (C_6H_6) in a separatory funnel recovering the clear bottom layer. No turbidity noted.

Euate Sediment from the $BaSO_4$ adsorption (100 mg/ml) of the above plasma No 126 washed 2x with dist water and then eluted for 10 min with 1/3 orig vol of 0.2M sod citrate. Aliquots of this eluate were (a) preserved frozen untreated (b) dialyzed 3 hrs against saline and (c) benzene extracted (b e) in a similar manner to the b e fibrinogen (see above). Another portion (d) was benzene extracted without dialysis and then (e) a fraction of this was dialyzed. Specific assays of components of eluates (a) (d) were obtained by the routine methods previously described. The data are given in Table VI and are expressed as a unitage/ml compared percentagewise with the statistical normal human plasma by which each test is standardized (cf Figure 16). These eluates (1) lack fibrinogen and thrombin (no clot in 3 days in control tests) (2) contain very small amounts of AcG (proaccelerin) but are rich in (3) prothrombin and (4) proconvertin. The dialysis and benzene extraction did not alter the prothrombin and proconvertin values significantly. The high ionic strength of the 0.2M citrate is inhibitory in clotting tests unless the eluate is highly diluted. It may be recalled that it is routinely diluted 50x in our eluate method III assays (p 59). Some incubations with 40 u/ml thrombin were performed with the undiluted eluates and failed to show any significant inactivation of the added thrombin. There was however a 2-3x lengthening of clotting times from the start as compared with a 0.85% NaCl control. With 0.2M sod citrate as the control however the clotting times (of the order of 25-30) were comparable. A more significant antithrombin assay was therefore conducted on the b e dialyzed eluate (e). The data are given in Table VII with details of method appended. There is no more than the merest trace if any thing of progressive increase in clotting times in the test (2) with the eluate which is nothing like the rapid activation in defibrinated plasma or serum or in (3) the untreated AcG preparation I used as a positive control (in Table VII) and compared (4) with a partly purified AcG⁺ described in Experiment 10 on p 76.

AcG Our regular $BaCO_3$ treated bovine serum (p 58) assays about 1200 percentage units of (pro)accelerin/ml. When assayed at full strength by the improved 2 stage method (p 59) it showed a barely assayable 1 unit/ml of thrombin yield. It is probable that traces of both thrombin and prothrombin persist in barium carbonate treated bovine serum but they are quite negligible in the dilutions usually employed. However we did attempt to purify this AcG preparation by an additional $NaSO_4$ adsorption. This reduced its AcG unitage to 900 percentage units/ml but the traces of thrombin and prothrombin persisted as shown by the control tests (1-4) in the experiments of Tables X and XI.

Ceph Cephalin was prepared from acetone dried dog brain [cf Eagle [110]] by several days extraction with ether removing the acetone soluble fraction and resuspending the dried acetone insoluble lipoids in saline as a stock 3% suspension uniformly dispersed with aid of a mechanical homogenizer. Dilutions (e.g. 0.5%) from this stock are stable and uniform. The stock 3% solution like other reagents is stored frozen at 20°C in the 'Deep Freeze'. This cephalin served as the standard in many experiments (e.g. p 98).

Ca The optimal $CaCl_2$ concentration

5 EXPERIMENTS WITH THE BENZENE EXTRACTION TECHNIQUE AND ITS RELATION TO 'AVAILABLE' CEPHALIN

Apart from the errors in his work on platelets and lung extracts C A Mills [335 336] correctly controverted Howell's view that calcium alone could activate prothrombin. Mills insisted on an essential role for cephalin acting along with calcium as a 'thrombokinas' in the sense of the Morawitz [345 346] theory (see p 8). His evidence [336] included the following experiment:

Dog plasma was precipitated at $\frac{1}{4}$ sat $(\text{NH}_4)_2\text{SO}_4$ for fibrinogen followed by $\frac{1}{2}$ sat with the ammon sulphate for a globulin fraction which served as a 'prothrombin' after both fractions were dialyzed free from excess salt. On extracting his prothrombin by shaking with benzene (C_6H_6) Mills obtained a preparation which when mixed with fibrinogen and calcium salt no longer gave a clot. Coagulation was restored however on further addition of cephalin.

Experiment 5 (1934) [123]

The present author's first modest essay into the blood coagulation field in 1934 was an attempt to confirm this experiment. We were able to do so however only after benzene extracting (b e) the fibrinogen also. With considerable difficulty the recalcified mixture of b e fibrinogen and b e prothrombin could also be induced to clot by (a) restoring the benzene extractives (recovered by evaporation) in a watery suspension of the mere trace of material recovered or (b) layering the benzene extract on the top of the test mixture.

Conclusion Of course an elementary knowledge of the lipid chemistry of the blood precludes any notion that this simple use of benzene can possibly extract more than a very small fraction of the fatty materials in the plasma fractions. Indeed the paucity of material in our evaporated extracts bears witness to this. Nevertheless the experiment does show the removal of some plasma property which can be restored by added lipoid (cephalin) and which would appear to fit the definition of a 'thromboplastin' (see later). Re-evaluation from the modern viewpoint raises at least one important possibility namely that the test materials are perhaps deprived of some labile clotting factor (? proaccelerin) not necessarily related to the partial lipoidal extraction. This possibility will be explored in the present thesis.

Experiment 6 (1956) Table VIII with assays on ELUATES (Tables VI VII)

Purpose To observe the clotting properties of an artificially isolated system of identifiable coagulation components after subjection to benzene extraction.

Reagents Fibrinogen (Fibr.) Precipitated at $\frac{1}{4}$ sat $(\text{NH}_4)_2\text{SO}_4$ from BaSO_4 adsorbed oxalated dog plasma redissolved in $\frac{1}{3}$ orig vol of saline (0.85% NaCl) and dialyzed against saline containing 0.005M sod citrate. Fibr. (b.e.): Fibrinogen shaken for 15 min at

Experiment 7 (1956) Table IX

Purpose To study further the clotting phenomena in 1 stage systems such as those of the preceding experiment and to test for clot promoting activity in the benzene extractives

Preparation of Extract (Extr) Preliminary observations gained from evaporation of the benzene layer from the preceding extractions indicated the extremely minute yields of extractives. It was therefore attempted to obtain a more significant amount of material from whole plasma. After 33 ml of a citrated dog plasma which had kept well for many months in the 'Deep Freeze' was thawed, filtered (from trace of sediment) and extracted as usual with an equal volume of benzene. Centrifugation of the top layer separated 30 ml of clear benzene from a small foamy deposit. The clear extract was evaporated to dryness in vacuo and the residue extracted with 3 ml imidazole buffered saline. Although this was definitely turbid, it did not contain a weighable amount of extracted material.

Method In a 1 stage test series similar to those of Experiment 6 the extract (extr) was compared with simple recalcification of eluate + fibrinogen mixtures. An optimal amount of cephalin was substituted in another parallel test.

As an independent observation the effects of b.e. AcG (a second extraction of the same reagent as used in Experiment 6 five days earlier) were studied both with and without cephalin.

Results These are given in Table IX. In (A) an eluate which had been benzene extracted and then dialyzed 5 days previously was used. In (B) this same reagent was submitted to a further benzene extraction and tested twice, namely (I) within 15 min. and (II) after about 3 hrs. Both series of tests employed the same freshly benzene extracted fibrinogen. Note the following:

- (a) simple recalcification times show a significant prolongation after the fresh benzene extraction B 2 vs A 2. However, there is some reversion to a shorter clotting time in the 3 hr. test of B 2 after standing several hours at room temperature.
- (b) the extr. reduces the clotting times, although not as well as cephalin does in the AcG poor systems.
- (c) AcG gives considerable aid to the clotting system but only moderately so with Ca only and much more so when a relatively adequate thromboplastic action is restored by cephalin.

All tests are consistently shorter in the A series. The precious extract was not tested with AcG in order to conserve it for the more significant 2 stage experiments to follow.

Conclusions Any conclusion from a 1 stage type of experiment must be tentative, but it does seem reasonable to conclude that at least four factors are contributing toward the observed clotting times.

was carefully determined for the eluate clotting tests by a preliminary series of mixtures corresponding to (8) in Table XI but with varied calcium concentration. The cited amount of CaCl_2 was found to be optimal.

Experiment Clotting test mixtures of fibrinogen (b e) and dialyzed eluates (as described above) constituted in essence a synthetic plasma system of known composition and free from overt inhibitors. Upon such the effects of benzene extraction could be studied in the clotting tests summarized in Table VIII.

Results The most significant finding was the much poorer clotting time (1005+) in (5) with the b e eluate activated with (added) Ca alone than in the same eluate (9) before benzene extraction (354). Moreover the + sign indicates the timing of the first wisp of fibrin and many more minutes were required for a solid clot in (5) whereas (9) progressed to a solid gel¹ within a minute or two. Recalcification in the presence of b e AcG (BaSO_4 treated) (7) considerably reduced the c t (210) showing that AcG lack was significant in the eluates as was known from the analyses in Table VI. Test (8) showed cephalin to be a good (? partial) thromboplastin in the presence of Ca and AcG. In comparison with (5) test (6) was equally significant in showing that cephalin could accelerate clotting even in a system very poor in AcG. In fact the 221 c t in (6) was better than the 354 c t in the simply recalcified untreated eluate (9). All four tests with the untreated eluate (9-12) were definitely better than the corresponding tests (5-8) with the b e eluate. The differences between 11 (107.5) and 7 (210'') are especially significant because both are performed in the presence of adequate accelerator factor. It should be noted that the 90 percentage units of AcG supplied in the 0.1 ml introduced into the total 1 ml mixture corresponded very well to the level normally encountered in plasma. Actually our synthetic system was hypoprothrombinemic (about 17 'percent') and hypoproconvertinemic (about 11 'percent') compared with a normal plasma. The 16.7 c t in (13) and 15.7 in (14) where tissue thromboplastin (Soluplastin¹) was added with AcG and Ca and the temperature raised to 37°C are of the order of magnitude that would be expected if we were testing a plasma of similar composition by the regular 1 stage 'prothrombin time' test. Tests 1-4 are essentially controls for thrombin and prothrombin in the AcG preparation. Thrombin is really absent from the eluate (see Table VI).

Conclusion These new experiments afford significant evidence of a diminished coagulability after benzene extraction. It is essentially independent of the presence or absence of abundant accelerator factor (AcG) although this has its own effect on clotting time. It is not an inhibitory phenomenon. It can be corrected in fact over corrected by the addition of cephalin or tissue thromboplastin. May it not therefore be concluded that (a) benzene extraction removes some thromboplastic¹ factor associated with the complex proteins of the clotting system and (b) benzene being a lipid solvent may not this factor be a thromboplastic lipid related to cephalin?

these systems particularly with Ca alone (2) The 'extract' adds a little which is significant and also indicates the extreme sensitivity of the test system (3) Although tests (1) and (2) are intentionally conducted with only the trace of contaminant AcG (see analyses in Table VII) in the eluate the provision of adequate accelerator in the form of 1:8 dil b e AcG still adds only a nominal increase in the trace of thrombin formation The important conclusion is that thrombin formation is extremely poor in a system adequate with regard to prothrombin, proconvertin, calcium and AcG but deficient in 'thromboplastin'

An incidental finding in these tests confirms the antithrombin tests of Table VII Note the excellent stability over 3 hrs of the very weak thrombins in tests (1) and (2) with eluate mixtures only whereas the 2 and 3 hr tests in the presence of the serum AcG are definitely beginning to lose potency despite the 1:50 dilution of its serum antithrombin There is some loss in all in 48 hrs especially in (3)

Experiment 9 is similar to the foregoing but relies upon the dilution and a little extra calcium to overcome the citrate The test data given in Table XI show

- 1) The AcG preparation has a minute amount of thrombin yielding potency even without any eluate (4)
- 2) Eluate with Ca yields very little thrombin even with the adequate provision of AcG (5) (7)
- 3) When the system is completed by addition of a 'thromboplastin' whether (a) cephalin or (b) crude tissue tpin thrombin formation is rapid and optimal
- 4) Cephalin is remarkably effective and compares well with tissue thromboplastin as to activity (shortest clotting time) attained but is definitely slower (7 min instead of 2-3 min) in reaching this optimum In fact the end point comparison in (6) and (8) indicates practically complete thrombin formation in the cephalin experiment
- 5) Extra proconvertin in (9) and (11) affords no benefits which agrees with the analytical findings (Table VII) showing that the eluate already contains it in adequate amounts

Conclusion The major conclusion from the foregoing experiments is that an extremely important clotting function must be assigned to factors playing a 'thromboplastic' role Significant among such is the phospholipid cephalin Modes of action need explanation however

6 EXPERIMENTS ON THROMBIN FORMATION

(6 A) REFERENCE STANDARDS

Experiment 10 (1956) Table XII Figure 14

Purpose To obtain reference assays for thrombin yields on a series of dilutions of 'standard' (canine) eluate containing prothrombin and proconvertin

Reagents Eluate (No 127) was prepared by 0.2M citrate elution of the usual BaSO₄ adsorbate from oxalated dog plasma as previously described (pp 55-71)

These factors are (1) eluate (i.e. prothrombin + proconvertin) (2) Ca (3) AcG (4) a thromboplastic type of factor which can be supplied rather well by cephalin and weakly by the benzene extract. The fact that benzene extraction does not result in a system completely unable to clot with calcium alone suggests that a small amount of some thromboplastic agent normally accompanies the reagents studied. That non clotting with Ca alone may be recorded in other systems of benzene extracted reagents (Experiment 5) may possibly be explained on two grounds viz (1) too great an ionic strength i.e. inhibitory to weak thrombin formation (2) antithrombic inhibitors in the system able to remove any small traces of thrombin formation before they can induce visible fibrin formation. The present synthetic system permits the detection of very weak thrombin formation by eliminating these two unfavorable circumstances.

In so doing however it still permits observation of a quantitative difference which can be interpreted as an ability of benzene extraction to remove a small portion of the potential 'thromboplastic' material in the clotting materials. From the nature of the extraction and the similarity to a weak cephalin in restoration of the clotting deficit may it not be surmised that we are dealing with a thromboplastic lipid? It is also suggested somewhat theoretically that the reappearance of greater clotting activity on simple recalcification at time after this has been lessened by benzene extraction may possibly indicate some 'free' form of lipid becoming liberated from the natural lipo protein complexes and being 'available' in this form for (a) the clotting reactions on the one hand and for (b) easy removal (in trace amounts) by the lipid solvent on the other.

Experiments 8 and 9 (1956) Tables X XI

Purpose To study the conversion of prothrombin to thrombin in variously activated eluates by a two stage test system with special reference to thromboplastic additives.

Reagents The same as those used in the preceding one stage experiments.

Method The 2 stage procedure is essentially that of our standard 'Method III' (see p 59). Note that the salt concentration (0.85% NaCl) and pH (7.3) are physiologically equivalent to plasma. Moreover the 50 fold dilution (0.1 eluate in 5 ml thrombic mixture) reduces the cit rate content to a point of non significance. However the chosen amounts of CaCl_2 are strictly optimal according to careful preliminary tests on rapidly activating test systems.

Experiment 8 is performed on dialyzed benzene extracted eluates: (1) with Ca alone (2) with Ca + extr (p 73) (3) with Ca + extr + AcG (b.e.)

The composition of the thrombic mixtures and the clotting test data after varying periods of incubation up to 3-48 hrs are given in Table X.

Conclusions (1) There is extremely little thrombin formation in

(6 B) ANTIHEMOPHILIC GLOBULIN IN RELATION TO THROMBIN FORMATION BY CEPHALIN AND BY TISSUE THROMBOPLASTIN

Experiment 11 (1956) Table XIII

Purpose To study the effects of purified antihemophilic globulin (AHG) on thrombin formation from eluate prothrombin in relation to cephalin and to tissue thromboplastin

Method Method III two stage procedure as in the foregoing experiments Standard volumes of reagents are given on p 60

Reagents Eluate and standard fibrinogen as in Experiment 10 The eluate provides prothrombin proconvertin and PTC (7 etc) AcG same purified preparation as described on p 76 used in 1:5 dilution AHG: a purified preparation of antihemophilic globulin from bovine plasma for which we are indebted to our colleagues in the Pathology Department Dr R H Wagner et al [488] Our assays for its very potent antihemophilic activity and for a trace of PTC are described in Experiment 34 on p 107 (Table XXXVII) The other specific assays showed no thrombin prothrombin or proconvertin and test (1) of Table XIII proves that it lacks thromboplastic activity It does contain citrate and a trace of fibrinogen The last causes a very small amount of clot when used in the 1:100 dilution of a 1% stock solution during the incubation of the thrombic mixtures It also interferes with tests for antithrombin Ceph: 0.1% diluted from the same 3% stock cephalin solution (p 71) as in the preceding experiments Tpln: 'Soluplastin' Schieffelin's (p 56) used (a) undiluted (1:1) or (b) at 1:5 dilution Ca: 0.15M CaCl₂

Results See data of Table XIII The control (1) without added thromboplastin but in the presence of AcG and AHG shows very little thrombin formation The trace activation is still continuing at 1 hr but is not significantly increased at 18 hrs (2) AHG with thromboplastin (1:1) shows little if any difference from the 'AHG free' control (3) i.e. the same maximal activation (15.9 c.t.) takes the same 3' time (4) AHG with thromboplastin (1:5) again shows no significant differences from the control (5) Both require 10 min for maximal activation and the slight difference in end-point clotting times is experimental and actually not in favor of the AHG containing mixture The trace of fibrin in the T.M. could be removing a little thrombin A very different result is seen in the cephalin experiments (6) with AHG and (7) without it There is a definite improvement in activation rate (7 min vs 15 min) and also a decidedly better (15.7 vs 25.5) thrombin yield when AHG is present

Discussion The circumstances in test (6) permit the interpretation that we are in basic principle performing by a new method the

Specific assays on Eluate No 127: (1) thrombin 0 (2) anti thrombin 0; (3) prothrombin 573 standard 2 stage (p 59) units per ml or 191 on basis of 'percentage' (of normal standard human plasma) (4) proconvertin 36 'percentage' units by 1-stage method (p 59) (5) AcG 1 stage (p 58): trace only

Fibrinogen (dog standard) was prepared from the BaSO₄ adsorbed plasma of the same dog by the usual technique (pp 55-70)

AcG† The routine AcG preparation (p 58) namely BaCO₃ adsorbed bovine serum was partially purified by precipitation at 1/3 sat (NH₄)₂SO₄ redissolving in original vol of saline and dialyzing 2 hrs against saline in cold room (4°C). A 1.5 dl of AcG† by preliminary test proved optimal for tests of Experiment 10

Assays of AcG† (1) The data included in Table VII (see p 71) show the very considerable removal of antithrombin by this procedure (2) specific AcG assay (1 stage p 58) shows considerable loss viz 555 'percentage' units per ml compared with an untreated sample of the original AcG preparation (1240 'percentage' units) but nevertheless a very serviceable preparation (3) no proconvertin; (4) merest traces of (pro)thrombin

Thromboplastin Schiefflin's 'Soluplastin' (p 56)

Ca 0.15 M CaCl₂ optimal in amount used

Method Standard Method III 'except for use of 1:5 AcG† and varying amounts of eluate. Hence: Saline and eluate (3.8 ml) 1mM buff (0.5 ml) 1.5 AcG† (0.1 ml) Soluplastin (0.1 ml) 0.15 M CaCl₂ (0.5 ml)

Results are given in Table XII showing the actual test data and graphically in Figure 14 plotting 'end point' (i.e. minimal) clotting times against relative strength of eluate. 100 'percent' in our standard technique refers to 0.1 ml eluate per 5 ml (total) thrombic mixture (T.M.). Since this represents a 50 fold dilution of the eluate it will correspond to 573/50 = 11.5 standard 2 stage prothrombin units per ml of thrombin forming mixture

Conclusions This assay series is very satisfactory for measuring differences in the thrombin yields between 150% and 1% ('percentage' units relative) of eluate. As the amount of prothrombin is reduced the thrombin yield as measured by the minimal clotting time endpoint is reduced (longer c.t.'s). The required incubation period (3 ± 1 min) is not significantly different however until the prothrombin is below 20 percent (of standard). With very weak e.g. 1 percent eluate the lengthening of the incubation period is significant. This might be due to the usual salt concentrations becoming unfavorable at very low protein (specifically prothrombin and proconvertin) concentrations. That relative excess of salts delays thrombin formation and lengthens clotting times is illustrated incidentally in Experiment 12 (p 79). Alternatively the lowered concentration of accessory factors (Stuart proconvertin) may be a sufficient explanation (see SUPPLEMENT)

(6 B) ANTIHEMOPHILIC GLOBULIN IN RELATION TO THROMBIN FORMATION BY CEPHALIN AND BY TISSUE THROMBOPLASTIN

Experiment 11 (1956) Table XIII

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Discussion The circumstances in test (6) permit the interpretation that we are in basic principle performing by a new method the

essential reactions involved in the 'thromboplastin generation test' (see p 47) Cephalin is an 'incomplete' thromboplastin and does not yield thrombin formation either as fast as or--in the present concentration (10γ/ml mixture) as completely as tissue thromboplastin. With the AHG (etc) however it does complete the thrombin formation to the same (15.7 sec c t) end point and in a time (5.7 min) between that of the 1:1 tpn (3 min) and that of the 1:5 tpn (10 min). It is presumed that PTC (and possibly other plasma thromboplastic factors see p 27) are available from the eluate. See Experiment 34 (Table XXXVII).

Conclusion This experiment gives some very important information about the thromboplastic effect of the phospholipid cephalin in that it indicates how a weak partial thromboplastin may be converted into a more powerful complete thromboplastin when AHG (antihemophilic globulin) participates in the reaction(s). There is evidence of some antihemophilic factor contaminant of our dialyzed eluate preparations (Table XXXVII Experiment 34). This could explain why cephalin acts as well as it does in test (7) for instance. It may very well be that this is the significant difference between our eluates and Seegers' more purified prothrombin (p 142) with respect to activation by Ca + cephalin.

Addendum Role of PTC? We do not at present have a suitable PTC preparation to investigate its probable participation (according to other current knowledge) in the generation of 'complete' thromboplastin. We may suggest however that it should be possible to study this in much the same way with the basic test eluate prepared from a very PTC deficient plasma. Some of our [298] human cases would be the most suitable source of such plasmas. It is just possible also that such might be prepared from a dog after administration of dicumarol, tromexan or similar drugs. We have some unpublished data on PTC lowering (cf [350]) in several human cases undergoing such therapy. Low levels of PTC were found in all such cases tested, but it is questionable whether low enough levels could be thus obtained for the suggested experiment without too much loss of prothrombin and proconvertin. Eluates from PTC deficient cases gave the answer (x SUPPLEMENT).

(6 B₁) THE THROMBOPLASTIN GENERATION TEST

We shall not include the experience we have ourselves had with the 'thromboplastin generation test' (p 46). We do not use it as a clinical routine because we believe our more specific AHG and PTC (and anti AHG and anti PTC) assays give more significant quantitative information. The thromboplastin generation test may be considered to have considerable practical value in differential 'spot' diagnosis of the hemophilic diseases. The test has a value in these disorders comparable perhaps to what the Quick (prothrombin time) test has in the different group of prothrombin and related deficiencies. There are a number of current workers both in England and in America who are experimentally studying variants of the thromboplastin generation test in order to learn more about the basic mechanisms and possible 'inter-

mediates' in these reactions (ref Bergsagel & Hougie [40] cited p 48) We are content therefore to leave to others this particular *line of inquiry* and merely to add our own confirmatory data to what is now widely known [354] and used namely that cephalin can substitute excellently for platelets (see p 47) in the performance of many variants of the thromboplastin generation test We shall however review some of our own experiments of many years ago and some more recent confirmatory data on certain aspects of the problem of 'intermediates' in thrombin formation with particular reference to the phosphatide cephalin and the role of calcium

(6 C) A CALCIUM CONTAINING 'INTERMEDIATE' IN THROMBIN FORMATION?

Experiment 12 (1956) Table XIV

Purpose To decalcify a thrombin forming mixture at various times from start of prothrombin activation and to observe the effects on thrombic activity Sequestrene Na_2 chosen as the decalcifying agent

Reagents Sequestrene Na_2 ('Versene' or EDTA) Alrose Chemical Co Providence R I has a molecular weight of 372 and solubility in water at 26°C of 11.1 g/100 ml 100 g EDTA 'sequesters' 10.5 g of Ca^{++} 3.72 g dissolved in dist water to 50 ml volume = 0.2 M (stock solution) EDTA: 0.1 M was used in Experiment 12 Elate (No 127) see p 75 AcGT partially purified see p 76 Ceph: 0.1% cephalin see p 71 Ca: 0.1 M CaCl_2 Fibr: Std (dog) fibrinogen see p 70

Method A special problem was presented by the high ionic strengths of the total salts in the mixtures used in Experiment 12 A compromise was made which permitted the obtaining of clotting times which were reliably readable Although prolonged by the high salt content they yet allowed the necessary control of the calcium

Thrombic Mixture 1.6 ml saline 1.0 ml imidazole buffer 0.2 ml AcGT (1:5) 0.2 ml ceph (0.1%) 1.0 ml eluate 1.0 ml CaCl_2 (0.1 M) 28°C

Series (A) 0.15 ml T M + 0.45 ml fibrinogen sequestrene (i.e. 8 vols fibr + 1 vol 0.1 M EDTA) This is properly controlled to be equivalent in all salts to the final test mixtures in series (B) Error

Series (B) After 5 min of incubation of T M (above) 1.2 ml was added to 0.6 ml sequestrene (0.1 M) and 0.2 ml samples tested on 0.4 ml (untreated) fibrinogen 'immediately' (15 sec) and at stated times thereafter

Series (C) After 68 min of incubation of T M (see above) another 1.2 ml was treated with sequestrene (0.6 ml) and 0.2 ml samples repeatedly tested as in (B)

Results Under these particular experimental conditions maximal thrombic activity was attained in the T M after 15 min incubation according to test series (A) in which the T M was incubated alone and the EDTA added (to control the salt mixture) only with the fibrinogen

In series (B) the initial 5 min test immediately after adding EDTA to the T M was only very slightly longer than the corresponding test of the (A) series. Thereafter however as incubation of the T M with the sequestrene continued thrombic activity was not merely arrested but actually underwent a very significant reversal to progressively longer and longer clotting times. In series (B) the initial test was if any thing slightly better than the minimal clotting time of series (A) but the small difference may have been due to use of a fresh tube of fibrinogen. On incubation with the sequestrene followed for an hour there was no trace of the 'reversal' phenomenon seen in series (B) but the T M retained its activity with excellent stability.

Discussion This experiment is a successful confirmation of an observation which the author first made in 1937 [128] using Howell type prothrombin + Ca + cephalin as the T M and repeated in 1947 [156] with a T M composed of Seegers' purified prothrombin + Ca + 0.25% tissue thromboplastin (from brain Squibb's). These data are reproduced on pp. 82-84. 1 M oxalate or 1 M citrate were used as 'decalcifying' agents. The former produced a heavy precipitate of CaC_2O_4 and the latter a translucency of clots which gave difficulties in the reading of end points. The clot timing was carefully performed however. The sequestrene experiments have good end points. An extremely important conclusion was drawn from the first presentation (1937) of the experiment namely that the results can only be explained by postulating an intermediate Ca containing 'complex' (of prothrombin + thromboplastic factor and we should now doubtless include the probability of the accelerator and converter co-factors also). This 'intermediate' is demonstrable only during the earlier phases of prothrombin conversion. After a time thrombin formation is complete and the 'ripe' thrombin can be decalcified without altering its clotting potency. Some evidence of a true reversal back to inactive prothrombin in the presence of excess of the decalcifying agent was allegedly adduced in the 1937 experiments (see pp. 82-83 but cf. p. 86).

Ignoring the citrate in the eluate (which isn't quite fair since it amounts to 0.04 molar in the 5 ml T M containing 1 ml of 0.2 M sodium citrate used in the eluate preparation) the following computation indicates the excess (equivalents) of EDTA over the added (1 ml of 0.1 M) calcium: In 3 vols of EDTA treated T M the 2 vols of T M supply $2/3 \times 0.02 \text{ M}$ (1/5 of 0.1 M) Ca while the 1 vol EDTA supplies $1/3 \times 0.1 \text{ M}$ sequestrene Na_2 . The relative molarities do not tell the full story however. Because of the ability of Ca to displace some of the Na in the chelating agent 100 g EDTA actually 'sequesters' 10.5 g Ca^{++} . Therefore the above amount of EDTA namely 24.8 milligrams per ml is actually enough to sequester 2.5 mgm Ca. The added Ca per ml of mixture however is only 0.53 mgm. Hence there is nearly 5 times as much sequestrene Na_2 added as would be required to remove all the added calcium even if the 0.04 M citrate played no part in the removal of Ca ions. Even a slight insufficiency of EDTA leaving a small part of the calcium still ionized results in failure to show the 'reversal' effect. Instead slow continued activation occurs in Series (B) under these conditions.

Conclusion These experiments support the hypothesis that an 'intermediate' containing loosely bound calcium is temporarily present during the activation of prothrombin to thrombin. The final (ripe) thrombin can be decalcified without loss of potency.

Experiment 13 (1956) Table XV

Purpose To repeat Experiment 12 but with the following modifications (a) use dialyzed eluate in order to provide more favorable ionic strength conditions and (b) substitute tissue thromboplastin for cephalin. Further to demonstrate whether the sequestrene reversed mixture can be re-activated by subsequent restoral of calcium etc.

Method Follows the same general principles as in the preceding experiment. Temperature: 28°C

Thrombic Mixture (T M) 7.2 ml saline + 1.0 ml imid buff + 0.4 ml AcG^+ (1:5 dil) + 0.4 ml 'Soluplastin' (1:10 dil) + 0.8 ml dialyzed eluate (No 127) + 0.2 ml CaCl_2 (0.1 M) Dial. El.: The dialyzed eluate assayed 90 percentage prothrombin units/ml and proconvertin 216 percentage units.

Series (A): 0.15 ml samples of T M tested on 0.20 ml fibrinogen + 0.05 ml 0.2 M sequestrene Na_2 at stated incubation intervals. Error!

Series (B): After 1½ min incubation of T M an 8 ml aliquot was added to a waiting 4 ml of 0.2 M EDTA and a second test series started at the 2nd minute now using 0.2 ml samples of mixture (B) to 0.2 ml untreated fibrinogen.

Series (C): After 1½ hr the remainder of mixture (B) was transferred to a Visking casing (cellophane) bag and dialyzed 1 hr against three changes of 0.85% NaCl. The casing membrane was tied so as to just enclose the material to be dialyzed with the result that there was no volume change during the dialysis. Subsequently a 3 ml aliquot was mixed with 1 ml of 'calcium activator mix' (Ca mix 3.0 ml imid buff sal + 0.8 ml AcG^+ (1:5) + 0.8 ml 'Soluplastin' (1:10) + 0.4 ml 0.1 M CaCl_2) and 0.25 ml samples tested against 0.2 ml of untreated fibrinogen after successive incubation intervals.

Series (D): As a control for test series (C) 0.08 ml dialyzed eluate was substituted for saline in a 1 ml mixture similar to the 'calcium activator mix' in proportions of other ingredients and diluted with 3 ml of buff saline just before adding the eluate and beginning the test series viz 0.25 ml samplings tested on 0.2 ml fibrinogen as in (C).

Results are given in Table XV. Notwithstanding the more rapid and complete activation (in about 5 min in (A) and in (D) series) by use of tissue thromboplastin the thrombin formation could be interrupted after 1½ min by adding 0.2 M sequestrene (1/3 total vol) and 'reversed' in a manner qualitatively indistinguishable from that observed in Experiment 12. The initial clotting time in the (B) series was 108.4. In an 8½ hr test (not included in Table XV) the c t was 323. The attempt (series (C)) to reactivate incubate (B) by (a) Ca addition (not illustrated) or (b) activator mix (Ca mix) was unsuccessful. Within the few seconds of experimental error all tests of the (C) series

reproduce the (B) c t value (at 90 min) with which the dialysis started. Only further c t lengthening (cf (B) at 185 min) was arrested. Nothing like the control series (D) was shown in (C).

Discussion Success in this rather tricky experiment depends upon careful consideration of (1) ionic strengths (total salt content) * (2) amount of sequestrene (3) rapidity of activation (depending upon amounts of AcG, thromboplastic agent, calcium and eluate in the T M) and (4) choice of a period during activation at which the EDTA can start its action before completion of thrombin formation, since ripe thrombin (Experiment 12) is insensitive to sequestrene inactivation. *In error, controls (A) of Expts 12, 13 got 1/4 instead of 1/3 EDTA.

Earlier Data The following data are selected from some 1937 experiments published by the author [128].

Experiment 14 (1937) Table XVI

Purpose To study the effects of excess oxalate at various stages of thrombin formation.

Method A two stage technique essentially similar to the foregoing.

Reagents Pro Howell type prothrombin from Berkefeld filtered citrated dog plasma (see pp 90-92). No attempt was made at the time to assay its prothrombin content and nothing was then known about proaccelerin and proconvertin. Ceph : 0.1% brain cephalin [126] prepared according to Howell [235]. Ca 0.1N (i.e. 0.05M) CaCl_2 . Oxal N/1 $\text{K}_2\text{C}_2\text{O}_4$. Fibr Precipitated from $\text{Mg}(\text{OH})_2$ adsorbed citrated dog plasma with $\frac{1}{4}$ sat $(\text{NH}_4)_2\text{SO}_4$, reprecipitated and again a third time now with $\frac{1}{2}$ sat NaCl. This fibrinogen was prothrombin free according to test with Ca + Ceph which gave no trace of clot at 38°C in 24 hours [126, 128]. T.M. 40 ml Pro + 4 ml Ceph + 4 ml Ca. Test 0.5 ml samples with 1.0 Fibr after various incubation periods 38°C .

Series (I) After about 11 min activation 10 ml T.M. was diluted with an equal vol. distilled water (control test) and incubated and tested parallel with (II).

Series (II) After 12 min activation another 10 ml aliquot of T.M. was mixed with an equal vol. of N/1 $\text{K}_2\text{C}_2\text{O}_4$ and 1 ml samples tested at intervals thereafter with 1 ml fibrinogen.

Series (III) After 4 hrs activation of T.M. a third 10 ml sample was treated with oxalate and tested in a similar manner.

Results These are given in Table XVI.

Experiment 15 (1937) Table XVII

Purpose To study the effects of excess citrate at various stages of thrombin formation.

Method and Reagents As in Experiment 14 except for substitution of N/1 sod. citrate (for the oxalate).

Results are given in Table XVII

Discussion (Experiments 15 and 16) The obvious differences between (1) 'fresh' thrombin (10-15 min incubated T M) and (2) 'ripe' (several hours old T M) in the progressive inactivation of (1) but not of (2) with a 50-100 fold excess of oxalate or citrate were noted [126]. The only effect on 'ripe' thrombin as in the initial (¼ min) test on 'fresh' thrombin is a prolongation of clotting time (compared with the control) due to the immediate effect of the high salt content.

The cited publication (p 758 of Ferguson [126]) includes mention of the following: By dialyzing 'fresh' thrombin which had been progressively inactivated by citrate to a clotting time of about 1 hour we have been able to check the inactivation and by recalcifying to restore the clotting time to a value almost identical with a control consisting of untreated prothrombin with a like amount of calcium and cephalin (Cf p 86).

Electrodialyzed 'ripe' thrombin (see p 65) was also unaffected by oxalate or citrate except for the immediate effect of the high salt concentration which was exaggerated by the low potency of these particular thrombin preparations.

The thrombin mixtures (T M) untreated were remarkably stable in these early experiments (p 760 of Ferguson [126]).

Conclusion (cited from [126]) It is clearly indicated that calcium forms an intermediary complex (prothrombin-cepahlin-calcium compound). This complex soon passes over into a stable thrombin. The intermediary can readily be deprived of its calcium with resulting inactivation whereas the final coagulant can be prepared calcium free without significant loss in potency. The mode of action of the so called decalcifying anticoagulants is now elucidated. In addition to their classical effect of preventing clotting by depression of the ionization of the calcium salts necessary for thrombin formation they can progressively remove calcium from the intermediary complex. Once the thrombin is ripe or fully elaborated however calcium is now no longer an essential component and oxalation or citration ceases to affect its potency except for the usual non specific immediate effect.

Experiment 16 (1947) Figure 15

Purpose To confirm the 1937 experiments (14:15) cited [126] but using one of Dr W H Seeger's purified prothrombin preparations and substituting a weak tissue thromboplastin for the phospholipid (cephalin). This 1947 experiment was presented graphically in three publications [156-141-147] and is reproduced in Figure 15.

Method Essentially similar to the foregoing.

Series 1 (T₁) 13.5 ml Seeger's bovine prothrombin (0.5%) + 0.75 ml tissue thromboplastin (0.25% Squibb's rabbit brain commercial preparation) + 0.75 ml 0.1 M CaCl₂. Clotting tests: 1 ml 1% bovine fibrinogen (Armour's) + 0.25 ml borate buffer, pH 7.7 (45 vol 2.5% H₃BO₃ 45 vol 0.5% NaCl 10 vol Na₂B₄O₇ 10 H₂O) + 0.25 ml T₁.

Series II: Same T M but tested (second phase oxalate control) by substituting 0.25 ml M/1 $K_2C_2O_4$ for the buffer

Series III (T_2) 3 ml T_1 5 min old + 3 ml M/1 sod oxal Clotting tests: 1 ml B F + 0.5 ml T_2

Series IV, (T_3) 3 ml T_1 30 min old + 3 ml M/1 sod oxal Clotting tests: 1 ml B F + 0.5 ml T_3

Experiment 17 (1956) Table XVIII

Purpose Further study of the progressive inactivation of thrombin forming mixtures using cationic exchange resin as the decalcifying agent Attempted reactivation

Method Essentially similar to the foregoing (see Experiment 13 p 81)

Reagents Same dialyzed eluate (No 127) 'purified' AcG^+ etc as in Experiment 13 0.1% Ceph (p 71) B F 1% Armour's bovine fibrinogen adsorbed with $BaSO_4$ (p 55) Dowex '50'--sulfonic acid resin (Dow Chemical Co Midland Mich) charged in sodium cycle (p 275 of Stefanini & Dameshek [453]) T M : 13 ml saline + 2 ml imid buff + 1 ml AcG^+ (1:5) + 1 ml 0.1% ceph + 1 ml dial eluate + 2 ml 0.02M $CaCl_2$ All clotting tests use 0.2 ml incubate + 0.2 ml B F at 28°C or (D) equivalent specified

Series (A): Control

Series (B): After 10 min incubation 10 ml T M is added to 1 g Dowex '50' and shaken vigorously at intervals thereafter with testing of clotting potency of the clear supernatant

Series (C): After 1 hr incubation another 5 ml of T M incubate is similarly treated with 0.5 g Dowex '50' and retested

Series (D): After 20 min shaking with Dowex '50' 2 ml of (B) is treated with 0.25 ml AcG^+ 0.25 Soluplastin (p 56) and 0.5 ml 0.02M $CaCl_2$ Thereafter tests are made with 0.3 ml of (D) + 0.2 ml B F

Series (E): Original eluate pre treated with Dowex '50' (100 mg/ml) for 1 hr (next day) and used in T M of same composition as (A)

Results Given in Table XVIII show that the decalcifying resin gives an excellent 'progressive' inactivation in (B) when the thrombin formation is not quite complete as seen from the control series (A) The similar tests after 1 hr (series C) show much less effect of the decalcification That a little progressive inactivation does occur in this series may be regarded as evidence that the thrombin formation is not yet quite complete This is probably due to the use of the relatively slow acting and 'incomplete' thromboplastic factor provided by the 0.1% cephalin with eluate poor in AHF

It was not possible to demonstrate any re activation by restoring Ca and other 'activators' in series (D) although these clotting times were somewhat shorter than in the (B) series at the start of the (D) experiment There is possibly a point of some minor significance in the data (e.g. first test in (B) and (C) suggesting a slight 'immediate' inhibitory action of the Dowex '50' which might be due to some alteration of the ionic strength pattern in these mixtures)

Series (E) performed next day clearly shows that Dowex '50 has no effect on the original eluate. After 1 hour's treatment with the resin the eluate activates just as well (if not better - 7 experimental) than in the control series (A) particularly reaching the end point in an identical (15 min) incubation period. In other experiments (see Table XX) bovine thrombin and dog fibrinogen were subjected to prolonged treatment with Dowex '50' without significant changes in their reactivity.

(6 D) A LIPID CONTAINING INTERMEDIATE IN THROMBIN FORMATION?

Experiment 18 (1956) Tables XIX XX

Purpose To study the effect of benzene extraction during the conversion of prothrombin to thrombin in eluates (prothrombin + proconvertin) activated by AcG cephalin and Ca salt.

Method Essentially similar to the preceding experiment and using the same preparations of dialyzed eluate, purified AcG† (1.5) cephalin (0.1%) and buffered saline Ca (0.02M). Armour's bovine fibrinogen (B.F.) 1% BaSO₄ adsorbed was used as substrate. 0.2 ml fibr + 0.2 ml (or equivalent) of test mixture after successive incubation periods.

Series (1): Control activation of the usual T.M. viz. 6.5 ml sal + 1 ml imid buff + 0.5 AcG† + 0.5 ceph + 0.5 eluate + 1.0 CaCl₂.

Series (2) After 5 minutes activation of (1) an aliquot was treated with an equal volume of benzene in a separatory funnel with vigorous shaking at intervals and 0.2 ml samples retested after further incubation.

Series (3) After 70 min inactivation of (2) a 1 ml sample was re-treated with an activator mix of 0.2 AcG† + 0.2 Ceph + 0.1 Ca and 0.3 ml samples tested thereafter.

Results are shown in Table XIX. The data clearly show a progressive inactivation in the b.e. mixture of incompletely formed thrombin (B). It was not possible to reactivate the 70 min sample of (2) either by the complete activator mix (C) or in other attempts (not illustrated) using (a) ceph alone or (b) ceph + AcG†.

Discussion This new experiment is a most interesting piece of evidence to support the 1936-1938 suggestion [126-129] that free or available cephalin also participates in the postulated intermediary during thrombin formation. The earlier benzene extraction experiments (pp. 70 et seq.) showed that fibrinogen eluate (prothrombin and proconvertin) and the AcG preparation could be shaken with benzene without more than minor changes in potency. Control tests on a bovine thrombin (i.e. fully formed thrombin) shown in Table XX likewise demonstrate the essential insensitivity of thrombin to the benzene extraction technique. With these controls therefore the progressive inactivation of partially formed thrombin (series (2)) is particularly significant.

Conclusion The foregoing evidence then definitely points to a loose binding of 'free' phospholipid (cephalin) into some 'intermediary complex' during the conversion of prothrombin to thrombin. Removal of such lipid by benzene extraction does not seem to restore the original prothrombin (activatable by Ca^{++} ceph. AcG) but to result in some inactive by product (? 'autoprothrombin' in Seegers' sense)

SUMMARY OF DATA ON THE POSTULATED 'INTERMEDIARY' COMPLEX (Experiments 12-18)

That there is a progressive inactivation not merely an arrest of further thrombin formation when a partially activated thrombin forming mixture is treated with a decalcifying agent has been repeatedly confirmed by experiments which we have made in 1937-1947 and again in 1956. In all we have used (a) three different types of prothrombin preparation (Howell type, Seegers type and our own 'eluate') (b) four types of decalcifying agent (oxalate, citrate, sequestrene and ion exchange resin) and (c) both partial (cephalin) and complete (tissue extract) types of thromboplastin. On the other hand the completely formed or 'ripe' thrombin is essentially devoid of calcium and is not specifically affected by the decalcifying anticoagulants. The progressively inactivated mixture cannot be restored to prothrombin (activatable by the usual means i.e. Ca^{++} thromboplastin, proconvertin, AcG). Earlier (p. 83) suggestion [128] that such reactivation might be possible must be reinterpreted as probably due (as was conceded at that time) to a considerable amount of the original prothrombin persisting unchanged through the test. We were always able to increase the potency of our electro-dialyzed thrombin by further recalcification for instance. The tentative suggestion is that the decalcified intermediary changes to an inert by product perhaps similar to Seegers' 'autoprothrombin(s)'.

The new (1956) experiments demonstrate an apparently similar progressive inactivation when a partially activated mixture (prothrombin + proconvertin + AcG + free cephalin + Ca^{++}) is treated with benzene whereas the final ('ripe') thrombin is unaffected by this lipid solvent.

These data support the hypothesis advanced by the author in 1937 namely that the conversion of prothrombin to thrombin involves an intermediary step during which the calcium and mobile phospholipid can be removed resulting in a progressive inactivation phenomenon. The new addition to this theory is the suggestion that the removal of either Ca^{++} or cephalin causes the intermediary to alter its normal reaction pattern (toward thrombin formation) and be diverted into some side reaction yielding an inactive by product (? autoprothrombin(s)).

(6 E) FATE OF PROCONVERTIN DURING THROMBIN FORMATION

Preliminary experiment In a repetition of Experiment 17 100 mg/ml Dowex '50' was added 5 min. after the start of activation of the usual (A) T.M. (same mixture as on p. 84). Just after the addition of the resin (B) the test clotting time was 20.9. After 1 hr. with the Dowex '50' it has lengthened to 106.4. Standard one stage proconvertin assays (p. 59) gave the following 'percentage values: (A) i.e.

untreated T M at start 110"; (B) i.e. 1 hr after adding Dowex '50' 547 This could mean merely that about half the original proconvertin was consumed (utilized) in forming the 5 min (? intermediate) thrombin and was surviving in the subsequent resin incubate The answer is certainly negative to the purpose of the inquiry namely to see if there was any increase in proconvertin activity as with some of Seegers' group's [13] experiments with 'autoprothrombin'

It remains for future investigation to explore further the possible connections between our line of approach and the quite different methods used in Dr W H Seegers' laboratories

(6 F) QUANTITATIVE RELATIONSHIPS OF CEPHALIN (VARYING CONCENTRATIONS) TO RATE AND YIELD OF THROMBIN FORMATION

When the prothrombin and proconvertin (in the eluate) the AcG c cium and saline buffer are kept constant our two stage Method III becomes a valuable quantitative technique for assay of thromboplastin according to the effects which their additions have upon the rate and yield of thrombin as determined by the clotting times (for a standard fibrinogen) in the second stage of the test Since these assays are relative it is necessary on each occasion to prepare a reference series of dilutions of some 'standard' at the same time and with all the same reagents as in the tests with the unknowns We choose as standards: (1) dog brain cephalin suspension (p 71) when testing various lipid preparations (2) 'Soluplastin' (p 56) when comparing various tissue thromboplastin preparations and (3) freshly obtained platelet suspension from a normal human donor (p 54) when assaying a patient's platelets for 'preformed' thromboplastic component (factor I in our listing p 50)

Experiment 19 (1955) Table XXI

Purpose To prepare a standard 'reference series' of cephalin dilutions and to study the effects on rate and yield of thrombin formation by 'Method III' as previously described

Reagents The prothrombin and proconvertin eluate is described on p 71 the untreated preparation being used in routine assays The AcG is described on p 58 and is further 'purified' (p 76) only for specially sensitive assays where it is desirable to minimize any instability of the end point The usual saline imidazole buffer (pH 7.3) and CaCl_2 are used

The standard T.M. (thrombic mixture) 3.7 ml saline + 0.5 ml imidazole buffer + 0.1 ml AcG (1.8) + 0.1 ml thromboplastic preparation (to be tested) + 0.1 ml eluate + 0.5 ml 0.15M CaCl_2 At successive incubation periods 0.2 ml T.M. is tested for the clotting times (at 28°C) with 0.2 ml fibrinogen (dog or bovine p 55)

Results are shown for an illustrative experiment in Table XXI

Discussion Some of the test series were not carried to completion

They are in fact a typical reference series taken at random from one of our routine studies in connection with investigation of the thromboplastic potency of various lipoids (see p 98) They include one or two small imperfections to illustrate the occasional experimental error e.g. test series 4 is 'out of line' By and large however they are reliable and serve to demonstrate the following:

(1) the smaller the concentration of cephalin the slower the rate of thrombin formation This is denoted by the sequence of clotting times and particularly by the incubation period required to reach the minimal clotting time end point

(2) the less the cephalin also the less complete is the thrombin yield This last is measured by the end point (minimal) clotting times in a relative manner and can be quantitated by use of reference standards of eluate dilutions such as those given in Figure 11 and Table XII

(3) while our cephalin is usually to be regarded as an incomplete thromboplastin especially when comparing activation rates with those in similar thrombic mixtures but substituting tissue thromboplastin (e.g. Experiment 9 p 75) the final thrombin yield in our test systems is often surprisingly good viz 90-100 percent in some cases and seldom under 50-60 percent if optimal strength cephalin is used For suggested explanation see p 78

(4) that there is an apparent 'optimum' cephalin concentration is illustrated in Table XXI by the poorer yield (28 l c t) despite the shorter (15 min) incubation period in the case of the 100γ strength (test 1) as compared with the 50γ (test 2) viz 22 'minimal c t' in 20 min incubation

That this is really due to the fact that cephalin is still a relatively crude preparation (see later) and contains some lipid inhibitor is now well recognized [73] Similar inhibition has also been reported for excess or certain fractions of tissue thromboplastin [4 91 358 359 193] and even for platelet suspensions [261] This immediately suggests however that our test can also be used to assay such antithromboplastic inhibitors This we do simply by mixing the unknown material with an optimal (usually 50γ) cephalin and noting the reduced thromboplastic potency

(5) The least cephalin concentration tested in this particular experiment was 0.78γ or 1:1 280 000 (test 8) This is still very much superior to the control (0) with no added thromboplastin It is obvious therefore that the method is extremely sensitive and is well able to detect thromboplastic activity in lipid suspensions (such as cephalin) diluted to something of the order of one in several millions The self imposed limits of the illustrated test series easily suffice for screening tests on unknown lipids such as those to be described in a subsequent section (p 98)

Conclusion These assays are an exceedingly sensitive measure of the relative thromboplastic potency of (cephalin like) lipids Alternatively used with cephalin they are equally sensitive indicators of antithromboplastic (anticephalin) inhibitors The cephalin concentration determines both the rate of prothrombin activation and (with sub optimal strengths of cephalin) the final thrombin yield

Earlier Data While the Howell type prothrombin used in our earlier research was not as good and as well controlled as in the present recent studies it did suffice to establish the same fundamental conclusions as shown in the following data reproduced from a 1938 publication [129]

Experiment 20 (1938) Table XXII

Purpose To study the effects of varying cephalin concentrations on the rate and yield of thrombin formation

Reagents Fibrinogen: Howell type prothrombin (Pro) Ceph and Ca(N/10) as described on p 82

Method Essentially the same type of two stage i.e. (1) activation and (2) testing with fibrinogen as in preceding experiments

T.M.: 10 ml Pro + 1 ml Ceph (cited final dilutions) + 1 ml Ca
Clotting Test: 1 ml fibr + 0.5 ml T.M. at successive incubation periods

Results are reproduced in Table XXII (Table 1 of cited ref. [129] in which the data are also presented graphically in its Fig. 2)

Discussion The results are essentially the same as those in Experiment 19. Instability of the end point was often troublesome with Howell prothrombins (see later) and there was the slight possibility that the differences persisting after 3 hrs. incubation might be partly due to instability complications. However this possibility could not explain the 4 hr. tests where after a 5 min. incubation with an additional 1/5 vol. of 1:1000 cephalin all series attained a minimum c.t. of 12. The extra dilution of the T.M. in all probability sufficiently accounts for the 12 value. It may be recalled that it was not until November 1939 that Mertz, Seegers and H. P. Smith [332] published the results of their experiments showing similar relationships of tissue thromboplastin. The two stage assays of prothrombin and thrombin gave much weight to the findings of these reliable investigators. However we continued to believe that relative clotting times in carefully controlled comparative experiments were equally valid evidence of the phenomena under consideration.

(6 G) EXPERIMENTS WITH TISSUE THROMBOPLASTIN

In 1948 we again performed the foregoing type of experiment but with highly purified prothrombin and using a tissue thromboplastin. The following data are reproduced from that work [156]

Experiment 21 (1948) Table XXIII

Purpose To study the effects of varying concentrations of tissue thromboplastin upon the rate and yield of thrombin formation from highly purified prothrombin

Reagents Prothrombin (Pro A) was prepared by ourselves from citrated dog plasma following closely the procedures (for bovine prothrombin) of Seegers Loomis & Vandenberg [431] It corresponded to 'Product 4' (stage of purification) of the cited authorities and compared very favorably with a number of Seegers' own (bovine) preparations with which we also experimented at that time [156]

Tpin A: Squibb's (commercial) rabbit brain thromboplastin
Fibr 1% B F (Armour's bovine see p 55)

Method 5 ml vol T M containing 2 ml Pro A + 0.25 ml 0.1 M CaCl_2 + tpin A (final concentrations stated in Table XXIII) in borate buffer pH 7.7 (see p 83) Clotting tests: 0.5 ml B F + 0.25 ml T M at $25 \pm 2^\circ\text{C}$

Results are summarized in Table XXIII stating (a) the final tpin concentration (b) the end point clotting times (sec) (c) the incubation period needed to reach the end point

Discussion Seegers type prothrombins at that time undoubtedly contained proconvertin but probably only questionable traces of proacelerin (AcG) [494] However these factors were not generally known at the time these experiments were performed It was merely a demonstrable fact that in our experiments Seegers type prothrombins could be activated very slowly under unfavorable activation conditions e.g. with Ca alone or sometimes without any addition [146] Cephalin was a poor activator of Seegers' prothrombin [146] Nevertheless the stability of our end points often for 3-4 weeks at room temperature in the bacteriostatic borate buffer solution was indeed remarkable The fact that small differences in the end point clotting times were apparent in the data (see Table XXIII) therefore did seem to confirm the fact that below a certain optimum 'lessening the thromboplastin definitely reduces the final amount of thrombin formed in addition to greatly slowing the rate of activation'

ADDITIONAL EXPERIMENTS WITH THROMBOPLASTIN tested on eluates (1956) qualitatively resemble the cephalin data (Experiment 19 Table XXI) and will not be cited in detail

Conclusion (Experiments 19-21) The author's data (1938-1948-1956) repeatedly confirm the apparent fact that the amount of thromboplastic factor (whether cephalin or tissue thromboplastin) below a certain optimum (which may be a matter of contaminant inhibitor) determines both the rate of prothrombin conversion and the final thrombin yield These ideas can be added to the 'intermediary theory' very nicely but it does not seem appropriate to assume that the relationships are 'stoichiometric' Even if this last could be demonstrated for an 'intermediate' it need not be true for the final thrombin

(6 H) SOME MODERN ASSAYS AND TESTS ON HOWELL TYPE 'PROTHROMBIN' WITH REFERENCE TO FACTORS WHICH MAY AFFECT THROMBIN FORMATION

Because of some uncertainties in retrospect concerning clotting factors other than prothrombin which may have been present or

deficient in our earlier preparations of Howell type 'prothrombin' (with which many of the fundamental observations presented in this thesis were originally made) it seemed desirable to subject this reagent to the scrutiny of our modern assay methods

Preparation As in the past (1937 1938) citrated dog plasma (p 54) was subjected to Berkefeld filtration (p 69) and subsequently defibrinated by warming cautiously in test tubes held in a water bath at 55°C for two minutes then filtering through coarse filter paper Prothrombin papers were prepared from 5 ml quantities of the filtrate by the acetone method of Howell [235] and his pupil Cekada [71] This consisted simply of rapid precipitation with an equal volume of acetone and immediate collection of the precipitate on a filter paper in a Buchner funnel under suction pump and quickly washing with a test tube full (about 20 ml) of ether and finally drying in an air current vigorously applied with the aid of a jet or electric fan

Prothrombin Solution (Pro) is obtained by cutting up the dry papers and extracting them with a suitable volume of distilled water (usually 10 ml per paper) containing a drop or two of 0.5% NaHCO_3 and subsequently filtering. The pH should be about 7.5 as tested with (a) phenol red indicator (b) pHydrion paper (Micro Essential Labs Brooklyn N Y) or (c) the (Coleman) glass electrode pH meter Borate buffer pH 7.7 (p 83) or imidazole buffered saline pH 7.3 (p 56) are superior extracting agents for the prothrombin papers. The former was used in the following experiment

Experiment 22 (1956) Tables XXIV A XXIV B

Purpose To assay the principal clotting factors in Howell type prothrombin

Methods (1) Fibrinogen test: 0.2 ml Pro + 0.2 ml thrombin (bovine Upjohn's 20 units/ml p 56) (2) Thrombin test: 0.2 ml Pro + 0.2 ml fibrinogen (p 55) (3) Prothrombin assay by improved two stage method (p 59) (5) Proaccelerin assay by specific one stage method (p 58) (6) Antithrombin test: incubate with thrombin as described on p 71 (Table VII) (7) (8) PTC and APTT assays p 107 Experiment 34 (cf Table XXXVII)

Reference Curves for the one stage assays were obtained on normal dog oxalated plasma dilutions and are shown in Figure 16

Results are shown in Tables XXIV A and XXIV B comparing the prothrombin (Pro) solution with the precursor plasmas

Fibrinogen: is absent from Pro (4) and (almost) from the heat defibrinated plasma (3) but of course is abundant in the original plasma (1) $ct = 9.3 \text{ sec}$ and in the Berkefeld filtrate (2) $ct = 10 \text{ sec}$

Thrombin: traces are not infrequently (but not invariably) demonstrable in Pro (4) In the cited test a small trace of clot appeared in 3/4 hr but was still very incomplete after many hours

Prothrombin: The normal (100%) about 254 two stage units/ml was found in (1) Nearly half was lost in the Berkefeld filtration (2) and in the heat defibrinated plasma (3) Only 10-15% levels were

found in the final Pro solution (4) Allowing for the double (cf plasma) vol of extract (10 ml per paper) the 'recovery' is about 20-29% of the original plasma prothrombin

Proconvertin: The original plasma (1) assayed a normal 96%. About 1/3 was lost in the Berkefeld filtration (2) and about 1/2 after the heat defibrination (3). A 20% level was found in the Pro solution (4) which correcting for the dilution represents a 40% 'recovery' of the original plasma proconvertin

Proaccelerin: The original plasma (1) assayed a normal 80%. This was reduced to 25% after the Berkefeld filtration (2) and negligibly more (22%) after the heat defibrination (3). The amount (25%) in the Pro solution (4) corrected for the dilution represents a remarkably good 50% 'recovery' of the original plasma proaccelerin

Antithrombin: The test in Table XXIV B showed this to be essentially absent

PTC: According to the assays reported in Experiment 34 (pp 107-108) (dog) Howell prothrombin (1:1) is better than a normal (human) plasma (1:5) in correcting a known PTC deficiency. These data suggest that the preparation contains most of the original plasma PTC

AHF: was also assayed in Experiment 34 (pp 107-108). It too is positive but in Pro (1:1) was less active than in 1:10 normal human plasma. The data on these assays must be considered very significant

Conclusion Howell-type prothrombin preparations are not only rich in prothrombin but also contain significant amounts of proconvertin, proaccelerin, PTC and AHF. They lack antithrombin and fibrinogen but may contain traces of thrombin. These findings may very well explain why the old experiments with Howell-type prothrombin gave significantly correct answers to a number of basic questions concerning the conversion of prothrombin to thrombin.

(6) QUANTITATIVE RELATIONSHIPS OF CALCIUM IN THROMBIN FORMATION

Experiment 23 (1956) Table XXV

Purpose To study the effects of decreasing calcium concentrations on the conversion of prothrombin to thrombin in dialyzed eluate (prothrombin + proconvertin) in the presence of adequate AcG and tissue thromboplastin

Method Two-stage technique similar to that used in previous experiments

T.M.: 0.2 ml eluate (No. 127 dialyzed) + 0.1 ml AcG† (p. 76) + 0.5 ml 'Soluplastin' + CaCl_2 (volumes of 0.02 M solution cited) + imid buff saline to total 10 ml vol. The stock (aqueous) CaCl_2 solution was 0.1 M and all dilutions were made with buff saline

Clotting tests: 0.4 ml fibrinogen (dog) + 0.2 ml 'adjusted' Ca (to give a final conc of 5 mM in the T.M. = F test mixture) + 0.2 ml T.M., tested after successive incubation periods: 26°C

Results These are given in Table XXV

Discussion The Ca conc was varied between 8 and 0.5 milli-Molar

in the T M keeping the other components constant The clotting tests were all conducted at a fixed (final) Ca conc of 5mM The effects of reducing the Ca concentration during thrombin formation were (1) a definite slowing of the earlier (e g 1 min) phases of activation but (2) no significant effect upon the final thrombin yield all c t end points were within a few tenths of a second of each other (4.5 ± 0.3 sec)

The negligible activation of the control (6) may just be the trace of (pro)thrombin in the AcG preparation as noted on p 76

Experiment 24 (1956) Table XXVI

Purpose To study the effect of increasing calcium concentrations on thrombin formation and to investigate whether there is a Ca optimum for this reaction

Foreword In the author's [129] 1938 experiments on this subject using Howell type prothrombin and cephalin as the thromboplastic agent the effects of the variable amount of calcium carried over into the final thrombin fibrinogen reaction (cf Experiment 2 p 65) were not controlled This led to some just criticism especially by Wohlsch [506] who cited Weitnauer's [499] controlled experiments In Experiments 23 24 25 we have avoided this error by adjusting the final Ca conc in the clotting test mixture to a fixed value This is conveniently done by including a carefully computed additional buffered Ca salt solution with the fibrinogen just before the final clotting test With high concentrations of calcium in the thrombic mixture (T M) this results unavoidably in some excess of Ca in all the final tests However this is a relatively minor point compared with the control achieved in validating the results of the main experiment

Method Essentially the same and with the same reagents as in Experiment 23 except for the increased amounts of CaCl_2 1 Molar stock (14.7% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) was dissolved in imidazole buffered saline

Results These are given in Table XXVI

Discussion These experiments were very carefully performed with excellent reagents and apparently homogeneous and only slightly opalescent mixtures Clotting times were accurately determined with a stopwatch and the end points were sharp The results however show some unexplained variability which is considerably more than in experiments at lower (calcium) salt concentrations We like to think our experimental error in clot timings is well within 10 percent and indeed it is usually within a few tenths of a second in literally thousands of tests in a variety of two stage systems We are not quite sure therefore whether the variability of the end points (minimal c t) in Experiment 24 has any real significance Accepting a $\pm 10\%$ experimental error the end points of I IV were all within 36 ± 4 sec and of V (at 45.4) just a little longer than these limits There was certainly no very striking and consistent reduction in the thrombin yield on increasing the Ca in the T M from 8 to 25 mM The 8 mM Ca level was best in this series (increasing Ca conc) as in the decreasing Ca series of Table XXV There is therefore some evidence of a

calcium optimum at this 8 mM level. Above this level the most consistent effects of increasing the Ca are the longer c t 's in the one minute test just as is the case (Experiment 23) in reducing the Ca conc below this optimum level. In the 5 min test however there appears to be some slight improvement with increasing Ca.

Conclusion It would seem that the main effect of varying the calcium level in our thrombin forming mixtures is to modify the initial rate of thrombin formation. Effects on the final thrombin yield are very questionable at Ca levels above the optimum and negligible (if any) at sub optimal Ca concentrations.

Experiment 25 (1948) Table XXVII

Purpose To study the effects of varying amounts of Ca^{++} during prothrombin activation using Seegers [431] 'purified' prothrombin in presence of tissue thromboplastin.

Foreword This experiment of the author's was published in 1948 [156]. In retrospect we were dealing with a very stable prothrombin undoubtedly containing proconvertin but very poor in AcG (hence the slow activation). The proper second phase controls were included.

Method and Results are given in Table XXVII. They demonstrate 1) no activation (in 2 hrs) in the absence of added Ca. 2) slower rate of activation if the Ca conc in the thrombic mixture is too low (2) or too high (4;5) but 3) no effect on the final (2 hr) thrombin yield i.e. identical (4 sec) minimal c t end points.

Conclusions (Experiments 23-25). The cited experimental data permit the important conclusions that the rate of thrombin formation from prothrombin in the presence of adequate thromboplastin and co factors (proconvertin and AcG) is determined by the calcium concentration in the activating (T M) mixture. Too little or too much calcium retards the thrombin formation and there is a definite Ca optimum at about 0.008 M in protein poor mixtures at pH 7.3 and 0.15 ionic strength. However there is no evidence that the final thrombin yield is affected by varying the Ca in the range studied. This could mean that any calcium even a trace could convert prothrombin to thrombin via the postulated 'intermediary' (p. 83) and then be released again to convert another portion of the prothrombin.

Effects of Traces of Calcium in Prothrombin Preparations Unless ion exchange resins are used it is extremely difficult to be sure that all traces of calcium are removed from any plasma fraction including prothrombin. There is always the question of protein bound Ca and the possibility of some free Ca^{++} released in attaining the equilibria studied by Nordbø [352]. We theorize therefore that the so called 'spontaneous' activation of prothrombin e.g. (1) in the experiments of Howell [233] and his pupil Cekada [71] and (2) in our own experience [156] with some of these and with Seegers' highly purified prothrombins are consistent with this ability of traces of Ca contaminant (and likewise traces of thromboplastin and co

factors) slowly but significantly to convert the prothrombin to thrombin by mechanisms which are identical in their essential nature with the processes going on in ordinary optimal activations

(6 J) THE QUESTION OF 'SPONTANEOUS' ACTIVATION OF PROTHROMBIN TO THROMBIN TRACE CONTAMINANTS? STABILITY OF PROTHROMBIN AND THROMBIN

Experiment 26 (1948) Table XXVIII

Purpose To investigate spontaneous thrombin formation in highly purified (Seegers') prothrombin

Method This experiment was actually the chance outcome of keeping a solution of Seegers' purified prothrombin (Prep H cited [146]) for 9 months in the ordinary (4°C) refrigerator and re-testing out of curiosity at the end of this period. It was found to be active thrombin. A dilution series (II) was prepared with the same amounts of calcium and tissue thromboplastin (not incubated) as had produced maximal activation of the original prothrombin solution 9 months earlier. At that time such a dilution series had been tested and the data (I) were available for comparison.

Results These are given in Table XXVIII. They clearly show identity of the thrombic potencies at successive dilutions with minor divergence only at the highest dilutions which are always subject to some experimental error. It is remarkable that as short a clotting time as 4 sec. accurately measured with a stopwatch has real meaning as a measure of thrombic potency. With such matching of serial dilutions it is an inescapable conviction that the two thrombins are indeed identical.

Conclusion It must be concluded therefore that any spontaneous conversion of prothrombin to thrombin may very well be due to trace contaminants of calcium and other activators. These trace amounts may be far too little to analyze. Nevertheless given sufficient time they may complete the conversion to a thrombin of potency identical with that ordinarily obtained after a short incubation period with optimal calcium and other activators. This obviously suggests that the calcium and other activators may be used over and over again intransferring the prothrombin bit by bit to the final end product. From one point of view this type of reaction may be regarded as 'catalytic'. This idea would seem difficult to apply to so simple an ion as Ca^{++} were it not for an explanation on the basis of an 'intermediary complex' such as we have argued for on the basis of a variety of other experiments.

Additional Considerations It is valid to draw from the above experiment the following further conclusions:

- 1) Prothrombin and thrombin are remarkably stable proteins in the absence of any factors which can alter them.
- 2) Our purified system is particularly free from inhibitors. It is possible to visualize a less pure system containing antithrombin for instance in which very slow conversion of prothrombin to thrombin

calcium optimum at this 8 mM level. Above this level the most consistent effects of increasing the Ca are the longer c t 's in the one minute test just as is the case (Experiment 23) in reducing the Ca conc below this optimum level. In the 5 min test however there appears to be some slight improvement with increasing Ca.

Conclusion It would seem that the main effect of varying the calcium level in our thrombin forming mixtures is to modify the initial rate of thrombin formation. Effects on the final thrombin yield are very questionable at Ca levels above the optimum and negligible (if any) at sub optimal Ca concentrations.

Experiment 25 (1948) Table XXVII

Purpose To study the effects of varying amounts of Ca^{++} during prothrombin activation using Seegers' [431] 'purified' prothrombin in presence of tissue thromboplastin.

Foreword This experiment of the author's was published in 1948 [156]. In retrospect we were dealing with a very stable prothrombin undoubtedly containing proconvertin but very poor in AcG (hence the slow activation). The proper second phase controls were included.

Method and Results are given in Table XXVII. They demonstrate 1) no activation (in 2 hrs) in the absence of added Ca. 2) slower rate of activation if the Ca conc in the thrombic mixture is too low (2) or too high (4;5) but 3) no effect on the final (2 hr) thrombin yield i.e. identical (4 sec) minimal c t end points.

Conclusions (Experiments 23-25) The cited experimental data permit the important conclusions that the rate of thrombin formation from prothrombin in the presence of adequate thromboplastin and co factors (proconvertin and AcG) is determined by the calcium concentration in the activating (T M) mixture. Too little or too much calcium retards the thrombin formation and there is a definite Ca optimum at about 0.008 M in protein poor mixtures at pH 7.3 and 0.15 ionic strength. However there is no evidence that the final thrombin yield is affected by varying the Ca in the range studied. This could mean that any calcium even a trace could convert prothrombin to thrombin via the postulated 'intermediary' (p. 83) and then be released again to convert another portion of the prothrombin.

Effects of Traces of Calcium in Prothrombin Preparations Unless ion exchange resins are used it is extremely difficult to be sure that all traces of calcium are removed from any plasma fraction including prothrombin. There is always the question of protein bound Ca and the possibility of some free Ca^{++} released in attaining the equilibria studied by Nordbø [352]. We theorize therefore that the so called 'spontaneous' activation of prothrombin e.g. (1) in the experiments of Howell [233] and his pupil Cekada [71] and (2) in our own experience [156] with some of these and with Seegers' highly purified prothrombins are consistent with this ability of traces of Ca contaminant (and likewise traces of thromboplastin and co

5) While this prothrombin solution was unstable in the sense that it slowly changed to thrombin on simple keeping yet it was amazingly stable in the sense of yielding the same potency thrombin and in the same incubation time when activated by Ca and tissue thromboplastin (two different preparations) in tests two months apart

6) Calcium alone gave an identical end point and it must be conceded that this although requiring a 4 day incubation is just as complete a thrombin formation. Hence it must be assumed that the 'purified' prothrombin still contains significant traces of thromboplastic and other necessary activators

Experiment 28 (1948) Table XXX

Purpose To study further the stability of prothrombin in the presence of Ca^{++} and thrombin

Method Since previous experiments had shown the very slow formation of thrombin on addition to highly purified (Seegers') prothrombin of Ca salt alone it was evident that such thrombin as was slowly formed would have every opportunity over several days to exert any possible effect on the still unaltered prothrombin. Were there any such effect it would be expected to show up in the final (end point) clotting times of test samples removed at intervals and rapidly (within 1 hr) activated by addition of tissue thromboplastin

T.M.: 15 ml 0.35% Pro E (Dr Seegers preparation assaying 13700 units per mg tyrosine N or 2450 units (2 stage) per ml of solution) + 5 ml borate buff + 1 ml 0.1M CaCl_2

Test Series (A): 0.5 ml 0.5% B.F. + 0.25 ml diluted T.M. 1 e 2 ml + 0.25 ml buffer to serve as control for (B)

Test Series (B): 2 ml T.M. removed after stated periods and treated with 0.2 ml thromboplastin (various tissue preparations) with successive testing of 0.25 ml samples on 0.5 ml B.F. (0.5%) until reaching the minimal clotting time end point noted

Results These are given in Table XXX. Note that in every case no matter what the age of the recalcified T.M. or which type of tissue thromboplastin was used the (B) series end points were all exactly 4 sec. and identical with that reached sometime between the 3rd and 8th day in series (A)

Conclusion This experiment completely fails to demonstrate any destruction or other effect of thrombin co-existing for days with its original (partly unchanged) prothrombin (cf [494]). These observations are important in ruling out possibilities of interfering reactions in the type of experiment relied upon for most of the data in the present investigations

(6 K) TESTING FOR (A) THROMBOPLASTIC AND (B) ANTI THROMBOPLASTIC ACTIVITY IN VARIOUS LIPOIDAL MATERIALS ESPECIALLY CEPHALIN AND SYNTHETIC PHOSPHATIDES AND THEIR CONTAMINANTS

Earlier Data (Table XXXII) Using T.M. containing Howell type prothrombin (cf Experiment 20 p. 89) data were obtained by the

may be proceeding but with the thrombin being removed as fast as it is formed by the action of antithrombin. Such a system would merely show a gradual loss of the ability of the remaining prothrombin to yield the original amount of thrombin in successive samplings tested after the conventional activation.

3) At the dilutions studied viz about 1000-2000 two-stage prothrombin (Seegers) units/ml the thrombin formed and in contact with the residual still unaltered prothrombin for weeks or months showed no evidence whatsoever either of (a) 'autocatalytic' acceleration of the prothrombin conversion or of (b) any destruction of the prothrombin by the thrombin (*cf* [494]). We have repeatedly mixed fully formed thrombin with a prothrombin activating system and failed to find any acceleration of the activation process with only one exception namely in the presence of platelets. This exception will be studied further in connection with the platelet experiments (p 109).

4) Either minimal traces or significant small amounts of thrombin could be detected in all 8 highly purified prothrombins viz 7 made by Dr Seegers and 1 by ourselves which were studied very exhaustively in 1948 investigations [156]. Some of these data bearing on stability questions will be reproduced as follows.

Experiment 27 (1948) Table XXIX

Purpose To investigate the stability of prothrombin in solution

Reagents Pro B: One of Dr W H Seegers' very highly purified bovine prothrombin preparations kindly provided for us with the information that it contained 15 200 units/mg tyrosine N or over 2000 units/ml of the 0.2% solution used in the present experiments. Tpln A Rabbit brain thromboplastin (commercial Squibb's) Tpln D Extract made with saline from frozen dog brain. T.M. 5 ml in borate buffer (p 83) pH 7.7 with additions as stated of 0.25 ml 0.1 M CaCl_2 with or without 0.25 ml thromboplastin. Clotting tests at $25 \pm 2^\circ\text{C}$ on 0.5 ml 0.5% B.F. (p 55) + 0.25 ml T.11 after stated incubation periods up to 7 days.

Results are given in Table XXIX

Discussion 1) Initial tests about 2 hrs after making up a 0.2% solution (in borate buffer) of the lyophilized prothrombin D showed a trace of active thrombin estimated to be less than 0.5% of the total potential thrombin yield (according to dilution tests given in the cited publication).

2) The relatively slow activations in these 1946 experiments we believe may very well be attributed to lack of sufficient proaccelerin.

3) The 1 min tests chiefly represent active thrombin in the solution since very little thrombin will be formed in this very short period under the slow activation conditions noted in (2). The first test in series (3) therefore indicates a considerable increase in thrombin contaminant spontaneously appearing in the 2 months storage at 4°C in the icebox.

4) Although the activation is much slower in (1) with Ca alone it finally (in 4 days) reaches a stable end point $\tau = 3$ sec identical with that reached in a couple of hours in the other two series.

Discussion (ref Table XXXII) The significant findings to date are:

1) Synthetic cephalins containing only saturated fatty acids e.g (tests 2 3 4) 1 2 distearoyl 1 2 dipalmitoyl or 1 3 dimyristoyl phosphatidyl ethanolamines (' α ' in the first two and ' β ' type in the third) are devoid of thromboplastic activity. This confirms earlier data by Grün and Limpächer [202] and Kabashima and Suzuki [263]

2) Efforts to synthesize cephalins containing unsaturated fatty acids are currently under way but not yet available for presentation in this thesis

3) A typical 'acetal' phosphatide but again with only saturated fatty acids viz stearyl acetal phosphatidyl ethanolamine (test 5 see p 98) is inert in our thrombin forming test systems so is the corresponding palmital compound (test 8) except for an extremely doubtful \pm

4) a myristal acetal phosphatidyl ethanolamine (test 6) which was 85-90% pure assayed quite inhibitory (anti thromboplastic). This looked like a possible lead toward the identification of a lipid anti thromboplastin. However when this synthetic compound was further purified it (test 7) lost the inhibitory properties

5) All efforts to run down the real inhibitor gave only the equivocal answer that it was residual in one or other trace fraction of the mother liquors (tests 9 12). None of the possible components which could actually be identified turned out to have significant effects in our test system and we are unable to suggest its true nature. There was a similar trace of inhibitor in the supernatants (fraction I test 17) in preparing the palmital acetal P lipid. Another trace contaminant (II) test 18 however gave only the negligible \pm test. Some of the trace fractions (tests 13 16) gave increased inhibition when added back to the purified phosphatide (No 7)

6) One possible lead resulted from these efforts namely that the trace contaminants (9 11 not 12) showing the most significant effects were mixtures of the less active ones. Indeed we were able to demonstrate this (15 16) by actually mixing two of the less inhibitory fractions. This enhanced inhibition by the mixtures suggests a possible synergism.

Conclusions These new efforts to identify (a) thromboplastic lipid(s) and (b) antithromboplastic lipid(s) via the approach of synthetic organic chemistry are still very preliminary. They do lead to the conclusions that

1) the ethanolamine base is not the primary determinant of thromboplastic activity

2) known cephalins containing saturated fatty acids only are inactive whether in the α or β form

3) acetal phosphatides do not have either thromboplastic or anti thromboplastic activity associated with their acetal structure

4) Anything more than this is speculative at the present time. Thus

5) it is merely a tentative hypothesis but not yet superseded that the physiological thromboplastic lipid must be either (a) a true cephalin of some specific type (?) containing unsaturated fatty acids) or (b) a contaminant or possibly (c) some metabolic by product of the known cephalin group of phosphatides

Hence this thesis will rest with the current status of this field of

author in 1943 on four fractions obtained by Dr J Folch of the Rockefeller Institute N Y in the re purification of brain cephalin [168] Following brief preliminary mention [138] we included these data in the publication of the Transactions of the Second Conference on Blood Clotting and Allied Disorders of the Josiah Macy Jr Foundation [144] They are reproduced in Table XXXI Data concerning fractions the percentage referring to yield in gm per 100 gm original 'cephalin' mixture V = phosphatidyl ethanolamine (15%); III = phosphatidyl serine (27%) I = inositol phosphatide (22%); IV = unidentified mixture (8%) In personal communications Dr Folch stated that III and I could contain about 5-10% of V (true cephalin) and IV 'may very well turn out to be either identical with or closely related to phosphatidyl ethanolamine' Thus the biochemical criteria were not fully adequate for a definite correlation with the thromboplastic activity qualitatively demonstrated by our very sensitive test which as shown in the table detected such activity in all four fractions although none of them was as potent as a weak buffered suspension of cat brain thromboplastin Moreover all the preparations supplied were dried and brownish from months of exposure to the atmosphere and must have lost a considerable amount of their thromboplastic potency Inconclusive as they are these tests nevertheless add some evidence to indicate that true cephalin is a potent thromboplastic phospholipid Any other conclusion will need further advance in the field of phospholipid chemistry

Current (1956) Data (Table XXXII) For the past few years the author has been using the preceding test system for the thromboplastic and antithromboplastic (anticephalin) assay of a number of synthetic phosphatides These agents have been prepared and chemically studied by colleagues Drs C E Anderson and C L Yarbrow of the Biochemistry Department at the University of North Carolina A report to the 1953 annual meeting of the North Carolina Academy of Science recorded the finding of neither such activity in the testing of an acetal phosphatide (test 5 Table XXXII) Tests on other current materials are listed as to results in Table XXXII

Method Our two stage (Method III) technique as previously described (p 59) Several dog eluates' (p 71) were prepared for use in these studies The AcG (118) see p 58 was a single batch as was the 'standard' (dog brain) cephalin (p 71) The 'unknowns' were supplied to us identified only by a code number and the descriptive data given to us by our biochemical colleagues only at the (temporary) conclusion of these investigations The following criteria were used for degree (+ or -) of activity

- (a) ++++ end point clotting times of < 20-30 sec (at $25 \pm 3^\circ\text{C}$) equivalent to > 10 γ /ml obtained in incubation periods of < 10-30 min
- (b) ±: slight enhancement of the std ceph but no significant thromboplastic activity alone
- (c) --: end point clotting times in 30 min with std ceph (50 γ /ml) so addition of over 5 min i.e. essentially complete inhibition
- (d) : end point ($\frac{1}{2}$ hr incub) c t of 3-5 min
- (e) - end point ($\frac{1}{2}$ hr incub) c t of 2-3 min
- (f) : end point ($\frac{1}{2}$ hr incub) c t of 1-2 min (< 0.1 γ)

tion With the amounts of agents tested in this particular experiment the heparin proved completely inhibitory to the cephalin (test 2) and practically so to the isolated phospholipid fraction (test 4) from the thromboplastin. Distinctly different was the result of test 6 in which the heparin showed no effect upon the tissue thromboplastin except in the 1 min test. Trypsin (see p 25) was also a good thromboplastic agent in these experiments (7) and resembled tissue thromboplastin in the very minor and early stage inhibition by heparin.

Even though the thromboplastin preparation (5) had been kept (at 4°C) for 3 days pending analyses and preparation of (3) it was distinctly more potent than its equivalent of the extracted P lipids.

Experiment 30 (1941) Table XXXIV

Purpose To demonstrate the significance of a heparin cofactor in its inhibitory effects during prothrombin conversion to thrombin [151a]

Method Similar technique to the foregoing

Reagents Pro: Howell type prothrombin (p 91) Tpln: Glass wool filtered saline suspension of frozen dog brain Ca: 0.1N CaCl_2 Hep: 5 Toronto units/ml diluted in saline from a Connaught Lab (Toronto) preparation kindly supplied through the courtesy of Dr C H Best. Stock solution in saline at pH 7.5 0.1%: 1 mg dry wt represented 110 Toronto units. Cofactor: This agent was a crude albumin obtained by precipitating citrated dog plasma between 50 and 100 percent saturation with $(\text{NH}_4)_2\text{SO}_4$ dialyzing away excess of salt and obtaining a final solution in 0.9% NaCl at pH 7.5. T.M.: 4 ml Pro + 5 ml cofactor (or saline in (1)) + 0.5 ml hep + 0.25 ml tpln + 0.25 ml Ca. Incubated at 75°C to avoid any deterioration. Clotting tests: Respective T.M.s (0.5 ml) added to 1 ml fibrinogen (p 82) + 0.5 ml of saline (tests 1-5) or saline mixture containing amounts of hep cofactor or both required to make tests 6-8 equivalent in the final clotting mixtures to 2-3-4 respectively. Tested at 38°C. Tests 5-6-7-8 were made with T.M. (1) at maximal (60 min) activation.

Results See Table XXIV. Note the following

Discussion (a) The chosen amount of heparin alone had a negligible effect on the fully formed thrombin (6) but did inhibit thrombin formation (2) in the early phases.

(b) This particular cofactor (albumin) was very slightly inhibitory to the 1 hr T.M. (1) in test 7 and to the later phases of T.M. (3). It was however slightly helpful to the earlier phases of activation in (3). In other experiments of a similar nature we have found either a little inhibition or a little aid in this early phase differing with various albumin preparations.

(c) The combination of hep + cofactor (4) was completely inhibitory to thrombin formation in tests extending over 2 hrs. This is the more significant because the test (8) on fully formed thrombin showed very little in the way of antithrombic action.

Conclusion A cofactor present in crude albumin fractions is

knowledge and continue to identify the thromboplastic phospholipid(s) as cephalin(s) for want of a better working hypothesis

Addendum Dr K M Brinkhous kindly supplied us with a few milligrams of Dr E Baer's (Connaught Labs Toronto) synthetic preparation [28 29] of 1,2 dimyristoyl-phosphatidyl α ethanolamine ('X') with which our Pathology Department colleagues [282] had reported a *feeble thromboplastic action*. In our routine tests a *thrombic mixture* which gave a minimal clotting time of a negligible 22 minutes (over 1 hr incubation) was able to give only a 6 min c t with 2.0 ml of 0.1% suspension of 'X' and barely improved to about 4 min c t with 2.0 mg of 'X' (per 5 ml T M). These are negligible results and point only to the most minute trace of something that can hardly be regarded as thromboplastic. We must conclude therefore that this compound like our own and others [202 263] is essentially devoid of true thromboplastic properties

(6 L) ACTIONS OF HEPARIN WITH SPECIAL REFERENCE TO THE THROMBIN-FORMING REACTIONS

Experiment 29 (1939) Table XXXIII

Purpose To study effects of heparin on the formation of thrombin from recalcified prothrombin in the presence of various thromboplastic agents [131]

Method The usual two stage technique similar to previous experiments. T.M.: 4 ml Pro (Howell-type p 91) + 0.5 ml CaCl_2 (0.05 M eq) + 0.25 thromboplastic agent (varied see below) + 0.25 dist water or heparin (0.5 mg equiv to 55 Toronto units)

Thromboplastic Agents

1) brain cephalin (p 71) 100γ per 5 ml T M
2) tpin Q a rabbit brain thromboplastin (dried preparation) prepared and kindly supplied by Dr A J Quick. The present solution analyzed Protein 104 mg % Total P lipids 11 mg % incl 4.6 mg cephalin

3) brain P lipid Quick's thromboplastin was extracted with alcohol ether (3:1) the acetone insoluble P lipids being recovered in petroleum ether dried and prepared in a 1:10 000 aqueous solution. The amount added in tests 3-4 represented the equivalent in phospholipid of the original tpin Q used in tests 5-6

4) crystalline trypsin 125γ a preparation kindly supplied by Dr M Kunitz of the Rockefeller Institute Princeton N J

The T M mixtures were incubated at a cool temperature 15°C in order to minimize any instability but clotting tests after the stated incubation periods were made at 38°C with 0.5 ml T M + 1 ml fibrinogen (p 82)

Results These are given in Table XXXIII

Data and Discussion Any cofactor (see later) which the heparin may need must have come from the Howell-type prothrombin prepara

Experiment 31 (1948) Table XXXV

Purpose To demonstrate that the mode of action of heparin in delaying the conversion of prothrombin to thrombin is 'anti thromboplastic' in nature

Foreword In the previously cited 1941 publication [151a] the author (with Dr A J Glazko) activated Howell prothrombin with Ca and tissue thromboplastin (in 1/1 1/2 1/32 dilutions) with and without heparin. The significant observation in the heparin experiments was that the rate of thrombin formation was delayed by the heparin the more the weaker the thromboplastin. The final thrombin yields (end point c t 's) were essentially similar however.

Another experiment [156] performed about 1948 with one of Seegers' purified fibrinogens essentially confirmed the earlier finding and was especially convincing because of the adequate controls. This will be reproduced as follows:

Method Two stage testing of prothrombin activation by method essentially similar to preceding experiments

T.M.: 4 ml Pro. E (ref [156]) + 0.5 ml borate buffer (p 83) in control (1) or 0.5 ml heparin (100 units) in series (2) + 0.25 ml tpin A (rabbit brain Squibb = 0.25%) + 0.25 ml 0.1 M CaCl_2 $25 \pm 2^\circ\text{C}$

Clotting-tests: (1a) 0.25 T M (1) + 0.25 buff + 0.5 B F 1" (p 55)
(1b) 0.25 T M (1) + 0.25 hep (10 unit) + 0.5 B F 17
(2) 0.25 T M (2) + 0.25 buff + 0.5 B F 17

Results These are shown in Table XXXV

Discussion The data shows:

- 1) complete activation to 4 sec end point in 8 hrs in (1a) with complete stability of thrombin for a week thereafter
- 2) some inhibition of the fully formed thrombin by the heparin as shown in (1b). This is very slight a mere $\frac{1}{2}$ sec (real) difference after completion of the T M activation. The smaller amounts of thrombin in the earlier phases of activation are more significantly inhibited (delayed) by the heparin however
- 3) inhibition of thrombin formation in the presence of the heparin is clearly shown by comparing (2) and its control (1b)
- 4) However the final end point although it took a week to attain is identical ($4\frac{1}{2}$) in the (2) and (1b) series

Conclusion Comparing this result with effects of a minor reduction in amount of thromboplastin (Table XXIII also reproduced from the 1948 paper [156]) the heparin inhibition of thrombin formation may be regarded as due to an interference with the action of the thromboplastin. Using a potent tissue thromboplastin the inhibition refers to a delay in rate of thrombin formation with no effect on the final thrombin yield.

Considerations Regarding Modes of Action of Heparin It should be emphasized that in these experiments no 'cofactor' was added and was unlikely to be present as a contaminant of the highly purified prothrombin. Further the heparin was added in relatively

therefore able very greatly to increase the inhibitory effect of heparin during thrombin formation even when the combined effects after full thrombin formation are almost negligible. These data support the view that cofactor is needed for the action of heparin in preventing prothrombin activation and this phenomenon is distinct from the established [242 382] antithrombotic action of heparin + cofactor which is exerted upon fully formed thrombin.

Addenda (I) Brinkhous Smith Warner & Seegers [67] in 1939 presented the first evidence that a serum cofactor is needed for heparin to prevent the conversion of prothrombin to thrombin. Their data may be briefly reviewed. One of Seegers' [435] early purified prothrombin preparations yielded 363 two stage units after 2 hrs activation by Ca and rabbit brain thromboplastin and practically as much (343 units) when heparin was also added. With heparin plus serum (cofactor) thrombin formation was blocked and almost all the prothrombin remained unchanged for two hours. With the serum alone all but 52 units of prothrombin were converted into thrombin in the same time period. This experiment shows incidentally that serum antithrombin acts only on fully formed thrombin. By and large this experiment proved the new observational fact. It is certain however that the two hour incubation period masks any effect which the heparin and serum alone might have on the rate of thrombin formation. Because of this doubt we performed the 1941 experiment [151a] cited above which we believe established the validity of the Iowa workers' conclusion beyond any reasonable question besides adding some new information as to what is going on during the activation period.

(II) The 'antithrombotic' action of heparin + cofactor was first observed in the original experiments of Howell & Holt [242]. Quick [382] found the cofactor to be associated with the albumin rather than the globulin in crude plasma fractions. Astrup [20] has reviewed the heparin studies made in the Carlsberg Foundation Laboratories (Copenhagen) including a 1939 confirmation of the Iowa discovery (above) and an important suggestion [21 22] that the cofactor necessary for turning heparin into an antithrombin which we have called 'thrombin coinhibitor' is a far more delicate substance than hitherto assumed. It disappears from plasma during clotting and is therefore not found in serum whether prepared by addition of thrombin by spontaneous clotting or by recalcification. It is further inactivated completely by heating to 56°C for 5 minutes.

(III) Since the topic of this thesis is primarily designed to explore the clotting functions of certain lipids (e.g. cephalin) and complex materials (thromboplastin) in which they occur, experiments with heparin are relevant when they deal with antithrombotic inhibitions of the thrombin forming reaction. To go into antithrombotic actions however would unduly extend our enquiry. We shall therefore omit some other wise valuable experiments e.g. (1) on heparin + cofactor as antithrombin [131] and (2) some unpublished data confirmatory of Astrup & Darling [22] which show by a different method that serum is devoid of heparin cofactor but because of progressive removal of added thrombin by the classical serum antithrombin (Astrup [20]) this thrombin becomes so weak in the serum mixtures that it is now very sensitive to the immediate inhibitory action of heparin in an effect which requires no cofactor.

3) added calcium and even better Ca^{++} cephalin greatly enhance the effects of trypsin Hence

4) the author's (with Mrs Betty Nims Erickson's) 1939 conclusion [149] that trypsin is not a thromboplastic agent in its own right (as Eagle & Harris [112] erroneously concluded) but merely makes Ca^{++} and 'free' cephalin available for the prothrombin activation presumably by releasing them from otherwise inert protein combinations Mrs Erickson and the author were never quite able to provide the convincing proof for this attractive theory and such is still unavailable However a 1948 experiment with B L Travis and E B Gerhelm (graduate students) ref [156] did seem to add some new evidence on the need for both calcium and thromboplastin in order to obtain the actions of trypsin in thrombin formation These experiments therefore will be recapitulated

Experiment 32 (1948) Figure 17

Purpose To demonstrate the need for Ca^{++} and thromboplastin in the trypsin activation of purified prothrombin

Reagents Pro. C (ref [156]) purified by Dr W H Seegers 0.3% solution Tryp Crystalline trypsin prepared by Dr M Kunitz [323] 40 units/ml Tpin. A Squibb's rabbit brain thromboplastin 0.25% suspension Ca: 0.1M CaCl_2 T.M. (thrombic mixtures):

- (I) 4 ml Pro + 0.75 ml borate buffer (p 8.3) + 0.25 ml Ca ;
- (II) 4 ml Pro + 0.25 ml buff + 0.5 ml try + 0.25 ml Ca ;
- (III) 4 ml Pro + 0.5 ml buff + 0.25 ml tpin A + 0.25 ml Ca ;
- (IV) 4 ml Pro + 0.5 ml try + 0.25 ml tpin A + 0.25 ml Ca

Clotting tests at 25°C on 0.5 ml B F (p 55) + 0.25 ml T M after successive incubation times (1 t)

Thrombin percentages were obtained by comparing the observed clotting times with a dilution series of maximally activated (24 hr) T M III The dilution data are plotted as $1000/c \cdot t$ (sec) in the dotted line of Figure 17

Results are depicted graphically in Figure 17 originally published [142] in the Ann N Y Acad Sci 1948 Note the following:

- 1) Ca alone produces a very slow activation complete in 50 hrs
- 2) Trypsin with Ca is not significantly better
- 3) $\text{Ca} + \text{tpin}$ gives adequate prothrombin conversion but is rather slow under the particular experimental conditions (? lack of AcG) being about 50% complete in 4 hrs and 100% in about 24 hrs
- 4) Trypsin significantly improves the $\text{Ca} + \text{tpin}$ causing the reaction to be over 90% complete within 1 hr and delayed (16-18 hrs) only in the penultimate stages

Conclusion Trypsin does not act alone or with calcium only but requires a source of thromboplastic agent With this further addition the enzyme has a significant effect in accelerating the thrombin forming reactions

high concentration. It may therefore be reasonably concluded that these are some special experimental facts pertaining to high concentrations of heparin (itself highly purified). They may have no real connection with a more physiological system in which a much smaller amount of heparin inhibits thrombin formation by acting in conjunction with a plasma cofactor. However similar results were obtained with physiological amounts of heparin in Experiment 47 p 120. The physiological significance of heparin has been reviewed on pp 30-31. Our researches have added a little to the understanding particularly of one of its possible modes of action namely to prevent prothrombin activation by functioning as an 'anti thromboplastin'. Study of this phenomenon in turn adds to the general knowledge of the thromboplastic mechanism and its very fundamental role in physiological thrombin formation.

We are endeavoring in this thesis to draw only those conclusions that are well supported by experimental fact and wish to avoid the orotical speculation as much as possible. In the heparin problems we are still on insecure ground and the following suggestions are very tentative but may be worth presenting as a possible lead for future investigations. Heparin (with its sulfuric acid moities) and cephalin (with its phosphoric acid groups) are both acidic substances known capable of combining with proteins and basic protein split products such as protamine (Chargaff & Olson [80]). May it not be possible therefore that the more acidic heparin can block or displace the essential thromboplastic phosphatide from certain protein combinations which are normally essential for the P lipid to participate in the process of thrombin formation (prothrombin activation)? May it not further be postulated that the cephalin is thereby deviated to some other protein say the cofactor altered (? made receptive) by the action of heparin? This could explain the 'antithromboplastic' action of heparin. The 'antithrombic' action in somewhat similar manner could be a deviation of thrombin to some cofactor + heparin complex (Astrup & Darling's [22] 'thrombin inhibitor') which according to Fitzgerald & Waugh (cited p 31) functions as a somewhat special type of antithrombin.

(6 M) THROMBOPLASTIC ENZYMES IN RELATION TO THROMBIN FORMATION BY THROMBOPLASTIC LIPOIDS AND (7) LIPOPROTEINS

Foreword 1939 Data. The thromboplastic action of crystalline pancreatic trypsin was briefly reviewed on p 25 and experimental evidence of this phenomenon was incidentally included in Experiment 4 (p 66) and Table V (on completely platelet free plasma) and in Experiment 29 (p 100) and Table XXXIII (on heparinized thrombin forming systems in which trypsin was substituted for tissue thromboplastin) much as Eagle (cited p 25) had substituted it for Ca + platelets. We shall not cite the experimental details but merely refer to the conclusions of our early work [150 149 136] viz

1) trypsin can clot oxalated or citrated plasma and activate (Howell type) prothrombin even in the absence of added calcium and cephalin

2) sufficient excess of the decalcifying anticoagulants can prevent these actions of trypsin

Addendum These and other studies in the cited paper [156] give some new insight into the possible significance of 'trace contaminants' in even the best modern prothrombin preparations. It is perhaps unfortunate that Dr Seegers has never seen fit to report on Micro Calcium (spectroscopic) and P lipid analyses of his prothrombin preparations but merely to assert (with reasonable pride) their 'purity on the basis of pot.ncy per weight of protein (or tyrosine N)

(6 N) PLASMATIC THROMBOPLASTIC COMPONENTS CONCERNED WITH THROMBIN FORMATION PTC AND AHF

Because thromboplastic function particularly of platelets is believed to be associated with a thromboplastin generating reaction (pp 46 et seq) in which PTC AHF and probably other factors are involved the following PTC and AHF assays were performed:

Experiment 34 (1956) Table XXXVII

Purpose To test for PTC and AHF in (I) dialyzed eluate (p 81) (II) Howell type prothrombin (p 91) and (III) Dr R H Wagner's [488] 'purified' AHG (antihemophilic globulin p 77) using (IV) a normal human plasma (D C F) as the factor control

Method Uses the specific one stage prothrombin assay (p 57) to test the improvement over saline (substrate control 0) of the prothrombin consumption of recalcified substrate plasmas (R J PTC deficient [294]; W B AHF deficient [152] human clinical cases)

Test 0.2 ml substrate + 0.05 ml additive are recalcified with 0.2 ml 0.025M CaCl_2 . After 1 hr in the case of the AHF tests and after $\frac{1}{2}$ hr in the case of the PTC tests (see Discussion) 0.05 ml of 0.1M sod. oxalate is added to check further prothrombin utilization. 1 stage prothrombin assays are performed on (1) the original mixtures and (2) the serum after oxalation and centrifugation (to remove clots). Amounts of additives are specified in Table XXXVII

Results Table XXXVII gives the experimental details and test findings. Using reference curves similar to those illustrated in Figure 16 (I) but obtained from human (instead of dog) standard normal plasma the 1 stage clotting times (c t at 37°C) are converted into the cited prothrombin (Pro) values expressed as percent of these normal standards:

$$\text{Prothrombin consumption (\%)} = \frac{\text{original pro (\%)} - \text{serum pro (\%)}}{\text{original pro (\%)}} \times 100$$

Discussion The PTC control (0) showed no clotting within 30 min (37°C) whereas all the additives caused clotting well within that period. At the time of these tests our substrate subject (R J) did show some consumption of prothrombin but gave significant assays when the incubations were restricted to $\frac{1}{2}$ hr. R J also presented another somewhat complicating problem in that he had a small amount of inhibitor (anti PTC [294 152]). This made his plasma less sensitive in our assays but still serviceable as the results indicate.

The AHF control showed a trace of clot in 22 min whereas the

Experiment 33 (1948) Table XXXVI

Purpose To study further the role of trypsin in systems of activating purified thrombin

Method Essentially similar to Experiment 32 including computation of 'percentages' (of maximal activation)

Reagents Pro. D (ref [156]) another of Dr Seegers' purified bovine prothrombins 0.2% solution Tpln. D saline suspension (decanted) from frozen dog brain (p 101) 0.1% Two amounts were tested Tryp Same crystalline trypsin 40 units/ml T.M.'s all contain 4 ml Pro. D + stated amounts of activators + borate buff to total 5 ml vol

Clotting tests: 0.5 ml B.F. (1%) + 0.25 ml T.M. incubated as stated 25°C

Results are given as 'percentage' activation in Table XXXVI

Discussion

- 1) With Ca only activation is very slow and measurable only after 1-3 hr but is complete (100%) in 48 hrs
- 2) With trypsin alone activation is slow and poor with best thrombin yield (20%) in 6 hrs The decline thereafter points to a possible thrombolytic action of trypsin
- 3) With Ca + trypsin a fairly good activation is noted with 100% completion in 3 hrs
- 4) With Ca + tpln at the higher concentration gave good thrombin formation complete in 30 min This was used as the reference standard
- 5) With Ca + tpln at the weaker (1/3) concentration was much poorer and required as long as (I) viz 48 hrs to reach 100%
- 6) With Ca + tpln (1/3 str) + trypsin the activation was much improved and complete in 3 hrs However this is only a little better in the earlier stages than in (III)

Conclusions (Experiments 32 33) Both the cited experiments prove that the enhancing (accelerating) effect of trypsin upon the conversion of 'purified' prothrombin to thrombin depends upon the presence of other activators namely calcium and thromboplastin. It is particularly when the calcium is adequate and the thromboplastin suboptimal that trypsin (experimentally) shows its most marked effects. It may be further concluded with some slight reservations because of the lack (and insuperable difficulty in obtaining) exact evidence that these highly purified prothrombins still contain traces of activator impurities. The differences in the results of the two experiments while slight may be significant suggestive evidence that (1) Pro. C (Experiment 32) contains insufficient trace of thromboplastin for the trypsin to add anything to the very minor effect (by and large) of added calcium whereas (2) Pro. D (Experiment 33) contains sufficient traces of some thromboplastin impurity for trypsin to exert a very definite effect with Ca. The further addition of the weak (1/3 str) tpln. D does not add much to the postulated thromboplastin contaminant so that its inclusion (series VI) gives little better activation than in (III).

imid buff sal before use Its proteolytic activity was tested in Experiment 44 (p 182) Plat 10x washed frozen and thawed dog platelets prepared October 29 1952 (1) and frozen stored at 20°C T.M. 5 ml mixtures in imidazole buffered saline containing 0.1 ml eluate 0.1 ml AcG (1.5 dil) + stated amounts of 'thromboplastic' additives + 0.5 ml 0.02M CaCl_2

Clotting tests 0.2 ml fibrinogen (dog) + 0.2 ml T.M. after successive incubation periods 27°C

Results These are given in Table XXXVIII and show the following

- 1) with platelets as the only thromboplastic additive thrombin formation is weak but unequivocal and yields a 30.3 c.t. end point in 30 min corresponding to about 35% activation of the prothrombin
- 2) with AHG a marked potentiation is seen in the 5 min and 10 min tests The trace of fibrinogen in the AHF preparation caused a small clot in T.M. (2) in about 4 min This was removed and the mixture remained clear thereafter It is possibly the adsorption of a little thrombin on this fibrin (removed) which explains the somewhat longer c.t.'s in the later tests of (2) The end point is at about 30 min as in (1)
- 3) a control with AHG only (no platelets) is very poorly activated but does yield a 164.2 c.t. in 30 min
- 4) with trypsin the potentiation is very marked Even after 1 min incubation a 155.5 c.t. was obtained whereas the other series (1) (2) did not give a clot in the 1 min incubate in over 2 hrs Moreover the end point (35.7) was definitely hastened to a 10 min incubation period
- 5) trypsin alone is very feebly thromboplastic with a trace of thrombin formation still continuing after incubation for 1 hr (445 →)
- 6) trypsin enhanced the minor effect of AHG (in absence of platelets) in 10-20 min incubations but the 30 min end point (172.6) was if anything a very little inferior to (3) Here again a trace of fibrinogen (less than in (2)) required removal after 7 min

Conclusion Antihemophilic globulin (AHG) is clearly able to 'potentiate' the weak thromboplastic activity of an old (over 3½ years!) well washed (x10) frozen stored (20°C) dog platelet suspension A trypsin solution (from a stock 2% sol in 50% glycerol + borate buffer) which had been preserved in the ordinary refrigerator for over 11 years was an even more striking 'potentiator' of the platelet activity The actions of AHG trypsin individually and in combination effect very little thrombin formation in our eluate mixtures containing prothrombin proconvertin PTC and added AcG + Ca but no added thromboplastin

Experiment 36 (1956) Table XXXIX.

Purpose To study the potentiation of platelet thromboplastin by (1) fibrinolysin and by (2) weak thrombin

Method This experiment was performed on the same day (May 24 1956) by the same technique as Experiment 35 (p 108)

Reagents The salts including 0.02M CaCl_2 eluate (dialyzed) AcG (1.5) and platelet preparation were the same and used in the same amounts as in Experiment 35

additives caused good clotting in less than 220 sec. The 10% (1 hr) prothrombin consumption in the AHF control (0) is a severe deficiency. All the materials tested showed the presence of both PTC and AHF in varying degree.

Conclusions Our 'prothrombin' reagents both (I) eluate and (II) Howell type prothrombin are quite rich in PTC. They also contain significant amounts of AHF about the same in the eluate and in the Howell prothrombin. In the case of the eluate this must mean that some AHG is co adsorbed on the BaSO_4 and resists the distilled water washings (p. 71). The traces of PTC in the 'purified' AHG may be due to the use of $\text{Al}(\text{OH})_3$ in the preliminary plasma adsorption. This is not quite as effective as BaSO_4 in removing PTC. That the detected amounts of PTC were only traces is evident from consideration of the concentration of the AHG tested (1%). In the AHC assays a 0.001% AHC addition gave an almost identical correction as 1/10 normal plasma which indicates about a hundredfold concentration of AHF in the stock 1% AHG solution.

Addendum Later another PTC deficient case (W.S.) presented the opportunity for re testing of the Howell prothrombin and the AHG preparation. This substrate in the control (0) showed a 37% prothrombin consumption in 1/2 hr at 37°C. A 1/10 normal plasma (D.C.F. p. 107) corrected this to 80% whereas the AHG (1%) gave 96% and the Howell prothrombin 100%. This is excellent confirmation of the Experiment 34 data on an inhibitor free substrate. It definitely confirms the trace of PTC contaminant in the AHF preparation.

7. TESTING OF CLOTTING FACTORS IN BLOOD PLATELETS

The clotting factors in blood platelets are reviewed on pp. 43-51. The following experiments contain some new information besides confirming some previously established facts. Tests on the enzyme and anti enzyme preparations used will be deferred to a separate section (p. 117) following the thrombin formation experiments.

(7A) THROMBOPLASTIC ACTION OF PLATELETS: POTENTIATION BY (a) AHG and (b) TRYPSIN

Experiment 35 (1956) Table XXXVIII

Purpose To study the thromboplastic action of platelets and the modifying effects of added (a) antihemophilic globulin (AHG) and (b) trypsin.

Method The two stage procedure on dialyzed eluate (see Table VI) as repeatedly used in previous experiments.

Reagents Eluate (No. 127) dialyzed as previously described (p. 81). AHC Dr. R. H. Wagner's [88] 'purified' preparation of May 9, 1956 described on p. 77. Tryp: 2% trypsin (Fairchild Bros. & Foster, N. Y.) in glycerol borate buffer (equal vols.) prepared Jan. 5, 1945 (I) and stored in refrigerator at 4°C. Dil. 1/100 with

ments of this era [476] which sought to relate platelet functions in the thrombin forming system with proteolytic enzymes and various anti proteases will be reproduced in the following

Reagents Some special testings of the enzyme and anti enzyme preparations will be deferred to a later section (p 117)

Borate Buffer 11.25 g H_3BO_3 + 4 g $Na_2B_4O_7$ 12 H_2O + 2.25 g NaCl + dist water to 1 liter pH (glass electrode) 7.7 + 7.75 specific resistance 170 ohms (at 21°C) effective ionic strength 0.055

Fibrinogen B F 1st borate buff sol of $BaSO_4$ adsorbed bovine fibrinogen (Armour's p 55)

Thrombins (a) Thr bovine thrombin (Upjohn's p 56) courtesy of Dr J T Correll A 100 units/ml 'stock' sol was prepared in borate buff (b) Thr* A special type of thrombin was prepared for one of the experiments and will be described in that connection (p 112)

Prothrombins Pro Several bovine prothrombins were made in our own laboratories following the directions of Seegers et al [431] However Dr Seegers kindly supplied us with a number of his own highly purified preparations Our [156] extensive studies using these products were reported in 1948 Excellent as Dr Seegers preparations were they did show evidence of trace impurities the control testing for which was an important part of our studies One of these trace contaminants was active thrombin In the tests to be cited it should be mentioned that no prothrombin solution was used (with rare deliberate exceptions which are stated) unless a preliminary test (0.5 ml B F + 0.25 ml Pro) yielded no trace of clotting in longer than 30 min Many of the experiments in the 1948 publication [156] were made before the establishment of our modern knowledge of the accelerator or co factors (proaccelerin or AcG syn factor V labile factor and proconvertin or SPCA syn factor VII stable factor etc) In the later experiments particularly those about to be cited [476] AcG was always added to our thrombin forming systems The only exception was when studying the role of this particular factor

AcG of bovine serum type was prepared and kindly supplied by Drs Ware and Seegers A 0.1% sol in borate buff showed traces of prothrombin and very minute amounts of active thrombin but these were considered to be non significant in the experiments cited It had no thromboplastic effects (author's tests)

Thromboplastins Tpln. A was prepared by ourselves from acetone dried dog brain a borate buffer suspension of which (0.25%) showed a high degree of thromboplastic activity Tpln. B Schieffelin & Co's Soluplastin (p 56) supplied through the courtesy of Dr E W Blanchard was used in some tests

Ca 0.5 M $CaCl_2$

Fibrinolysin Lysin: a purified enzyme preparation from beef serum [302] supplied through the courtesy of Dr E C Loomis (Parke Davis & Co Detroit) 1st sol in borate buffer was prepared immediately before use In later work we used our own dog fibrinolysin [291]

Antifibrinolysin Afln a purified fraction from beef serum also supplied by Dr Loomis [303] in November 1947 A 0.4% extract was made with borate buffer and centrifuged from a considerable residue of insoluble material

Trypsin Tryp. A Crystalline trypsin (from pancreas) prepared

Lysin A 43 month old preparation of (dog) serum fibrinolysin described on p 118 The 1:40 borate buffer dilution of this lysin was taken from frozen storage (20°C) thawed and used in the experiment i.e. 0.1 ml per 5 ml T.M.

Thr Bovine thrombin (Upjohn's p 56) 0.1 units/ml in buff. saline freshly prepared clotted an equal (0.2 ml) vol. of test fibrinogen (dog) in 6 min. At the 1:50 dilution in T.M. (7) therefore there was not more than 0.002 of a thrombin unit per ml. This extremely small amount could not possibly add its own direct effect to the observed clotting times. The control (9) without platelets or enzymes serves for both Experiments 36 and 35.

Results are given in Table XXXIX and show the following. Compared with (a) the negligible traces of activation (9) in the absence of platelets and enzymes (which are practically without effect alone) and with (b) the test (1) of Experiment 35 with platelets alone both the aged fibrinolysin (8) and the very weak thrombin (7) give evidence of a significant potentiation of the platelet thromboplastic action. That this is not more marked (as in Experiment 37 p 112 for instance) is probably due to our use of unnecessarily weak additives. Nevertheless the considerably shorter c.t.'s in the 5 min. test are quite meaningful.

Conclusion Fibrinolysin and thrombin therefore are two other agents with which we are able to demonstrate a 'potentiation' of the thromboplastic action of platelets. Considering the age (over 3½ years) of the platelet preparation a remarkable stability of the platelet thromboplastin is manifest.

Older Experiments In 1936 the author [126] performed some clotting experiments with platelets extracts of the cornea and lens of the eye (chosen because of absence of a blood supply) and prothrombin activated by Ca and cephalin. In retrospect these data do not seem to merit inclusion among the much more definitive types of experimentation in the present thesis except for mention of the fact that they led to the formulation of a cephalin availability theory (see later) and the suggestion that prothrombin (the protein factor of the coagulant) may occur in platelets, cornea and lens as the possible result of diffusion from the blood stream via the lymph and tissue fluids in the case of the eye tissues.

In the next decade only a few experiments were made with platelets (substituting for cephalin) in the activation of Howell type prothrombin in the presence of calcium. These did not seem to offer anything new. Eagle & Harris' [112] experiments (1937) which suggested that trypsin could coagulate blood by the same effect as the physiological system Ca plus platelets (or Ca plus tissue extracts) inspired the present writer to an extensive series of studies with trypsin and later other proteolytic enzymes including fibrinolysin (plasmin or tryptase ref [143]) in an endeavor to learn more about their possible relations in the blood clotting system.

In the period 1947-1949 and subsequently many experimental testings of platelets in artificial clotting systems were made by the author and his graduate students notably B. L. Travis. Some of the experi-

Lysin A 43 month old preparation of (dog) serum fibrinolysin described on p 118 The 1:40 borate buffer dilution of this lysin was taken from frozen storage (20°C) thawed and used in the experiment i.e. 0.1 ml per 5 ml T.M.

Thr Bovine thrombin (Upjohn's p 56) 0.1 units/ml in buff. saline freshly prepared, clotted an equal (0.2 ml) vol. of test fibrinogen (dog) in 6 min. At the 1:50 dilution in T.M. (7) therefore there was not more than 0.002 of a thrombin unit per ml. This extremely small amount could not possibly add its own direct effect to the observed clotting times. The control (9) without platelets or enzymes serves for both Experiments 36 and 35.

Results are given in Table XXXIX and show the following. Compared with (a) the negligible traces of activation (9) in the absence of platelets and enzymes (which are practically without effect alone) and with (b) the test (1) of Experiment 35 with platelets alone, both the aged fibrinolysin (8) and the very weak thrombin (7) give evidence of a significant potentiation of the platelet thromboplastic action. That this is not more marked (as in Experiment 37 p 112 for instance) is probably due to our use of unnecessarily weak additives. Nevertheless the considerably shorter c.t.'s in the 5 min. test are quite meaningful.

Conclusion Fibrinolysin and thrombin therefore are two other agents with which we are able to demonstrate a 'potentiation' of the thromboplastic action of platelets. Considering the age (over 3½ years) of the platelet preparation, a remarkable stability of the platelet thromboplasticity is manifest.

Older Experiments In 1936 the author [126] performed some clotting experiments with platelet extracts of the cornea and lens of the eye (chosen because of absence of a blood supply) and prothrombin activated by Ca and cephalin. In retrospect these data do not seem to merit inclusion among the much more definitive types of experimentation in the present thesis, except for mention of the fact that they led to the formulation of a cephalin availability theory (see later) and the suggestion that prothrombin (the protein factor of the coagulant) may occur in platelets, cornea and lens, as the possible result of diffusion from the blood stream via the lymph and tissue fluids in the case of the eye tissues.

In the next decade only a few experiments were made with platelets (substituting for cephalin) in the activation of Howell type prothrombin in the presence of calcium. These did not seem to offer anything new. Eagle & Harris' [112] experiments (1937) which suggested that trypsin could coagulate blood by the same effect as the physiological system Ca plus platelets (or Ca plus tissue extracts) inspired the present writer to an extensive series of studies with trypsin and later other proteolytic enzymes, including fibrinolysin (plasmin or tryptase ref [143]) in an endeavor to learn more about their possible relations in the blood clotting system.

In the period 1947-1949 and subsequently many experimental testings of platelets in artificial clotting systems were made by the author and his graduate students, notably B. L. Travis. Some of the experi-

same prothrombin preparation in borate buffer which had been allowed to stand with CaCl_2 for several weeks in the refrigerator (4°C). Tests showed that it was completely converted into thrombin since incubation with thromboplastin and AcG gave no further enhancement of its clotting potency. Such a preparation seemed well chosen to avoid the introduction of any factors except the thrombin unless per chance there are some unknown factors in the (recalcified) prothrombin itself. With these unsupported reservations we must conclude that weak thrombin can act as a remarkable potentiator of platelet thromboplastin. Since AcG is added this would not appear to be due to the known effect [492] of thrombin in converting proaccelerin to accelerin. Attempts were made to run controls without platelets. These showed only insignificant shortening of the clotting times in the parallel test (O) without activators (except for the Ca). This cited test (O) merely showed the trace of thrombin in the freshly prepared prothrombin solution and this could not be correlated with the weak thromboplastic action of the platelets shown in (1). The added thr* (p 111) (diluted) clotted a test fibrinogen in 750 sec when freshly prepared but rapidly lost potency on standing in glass.

The fibrinolysin test (3) showed a potentiation of the platelet thromboplastic effect qualitatively resembling that of thrombin and even quantitatively similar in this particular experiment. It is difficult to test a fibrinolysin for traces of thrombin because the enzyme lyses the test fibrinogen before it has a chance to clot with a very weak thrombin. We did perform this control test however in the presence of antiproteases (esp antifibrinolysin) which seemed to rule out this possibility. It is not possible therefore to deduce a common mode of action for these two dissimilar agents (thrombin and fibrinolysin) with any factual knowledge currently available.

Conclusion We present the experimental facts therefore which *prima facie* indicate that (1) platelets have a weak thromboplastic action in systems such as those described and that (2) weak thrombin and (3) serum fibrinolysin each have a significant potentiating action on this platelet effect. Experiment 37 was even more satisfactory and controlled than the recent confirmatory Experiment 36.

(7 B) EFFECTS OF ANTIPROTEASES ON VARIOUS 'THROMBOPLASTIC' ADDITIVES IN THROMBIN FORMING SYSTEMS

Preliminary Studies: These will be cited in later sections. These clearly established the following facts needed for interpretation of the thrombin forming experiments.

I None of the antiproteases had any inhibitory action on the thrombin fibrinogen reaction (see Experiment 43 p 117 and Table XLVI)

II In higher concentrations than those selected for the present experiments trypsin was thrombinolytic and Loomis' fibrinolysin very feebly so requiring several days incubation in order to demonstrate this.

III Again in too high enzyme concentrations to be significant in the present experiments a question of prothrombinolysis might arise 430 301. However Dr Lewis Ann C Howe and the author [299]

and supplied by Dr M Kunitz [323] (Rockefeller Institute Princeton N J) whose 'units' of activity are cited [353] Tryp. B a very satisfactory commercial trypsin (Fairchild Bros & Foster N Y) of which a stock 2% solution was made in 50% glycerol and borate buff (i e equal vols)

Anti proteases (Trypsin inhibitors) For antifibrinolysin see above P.I. pancreatic inhibitor (anti trypsin) highly purified (Lot P-A 97-1) Sharp & Dohme Labs courtesy of Dr L A Kazal (cf [353]) S.B.I. soybean inhibitor a crystalline preparation kindly supplied by Dr M Kunitz [272] (see above) N.B.I. a re purified navybean inhibitor (see p 117) kindly supplied by Dr D E Bowman [59] (Indiana University)

All these antiproteases were prepared in a 0.1% solution in borate buffer. The S B I was diluted to 0.01% before use. Special testings of the antiproteases will be cited later (p 117)

Platelets (Plat.) cf p 54 Well washed platelets were obtained as follows 100 ml dog blood was collected into 13 ml of 3.4% trisod citrate. Repeated centrifugings for a few minutes at 1000 r p m in the International Refrigerated Centrifuge were performed until red cells were no longer visible in the sediment. The 'platelet rich' plasma was then centrifuged for 30 min at 5000 r p m and the platelet sediment resuspended and well washed at least three times by thorough mixing with successive 50 ml lots of 0.9% NaCl containing 1/8 vol 3.4% sod citrate (we later used Triton 'p 54) and a final washing in plain saline. The final suspension concentrated in 1-2 ml saline was examined microscopically. Wright stained smears revealed only granular platelet material with no leukocytes and only a rare erythrocyte.

Experiment 37 (1949) Table XL

Purpose (as in Experiment 36) To study the thromboplastic action of platelets and its potentiation by (A) weak thrombin and (B) fibrinolysin

Method A two stage system similar to previous testings of thrombin formation

T.M.: 5 ml vol containing (with borate buff) Pro + AcG + Ca + plat with and without thr.* (p 111) or lysin

Clotting tests at 26°C 0.5 B F + 0.25 T M

Results These are shown in Table XL

Discussion This experiment is one which we have many times repeated to show the much better thrombin formation in a system of purified Pro + AcG + Ca + platelets when a tiny trace of thrombin is also added (test 2). It must remain an open question whether a test system completely devoid of thrombin (even minutest traces) might someday be obtainable and fail (?) to activate with platelets and Ca (in the presence of proconvertin AcG etc). Pending such a finding it seems to be established that platelets are an 'incomplete' thromboplastic which can be potentiated by thrombin. The thr.* used in Experiment 37 was a special preparation. In fact it consisted merely of the

same prothrombin preparation in borate buffer which had been allowed to stand with CaCl_2 for several weeks in the refrigerator (4°C) Tests showed that it was completely converted into thrombin since incubation with thromboplastin and AcG gave no further enhancement of its clotting potency Such a preparation seemed well chosen to avoid the introduction of any factors except the thrombin unless per chance there are some unknown factors in the (recalcified) prothrombin itself With these unsupported reservations we must conclude that weak thrombin can act as a remarkable potentiator of platelet thromboplastin Since AcG is added this would not appear to be due to the known effect [492] of thrombin in converting proaccelerin to accelerin Attempts were made to run controls without platelets These showed only insignificant shortening of the clotting times in the parallel test (O) without activators (except for the Ca) This cited test (O) merely showed the trace of thrombin in the freshly prepared prothrombin solution and this could not be correlated with the weak thromboplastic action of the platelets shown in (1) The added thr* (p 111) (diluted) clotted a test fibrinogen in 750 sec when freshly prepared but rapidly lost potency on standing in glass

The fibrinolysin test (3) showed a potentiation of the platelet thromboplastic effect qualitatively resembling that of thrombin and even quantitatively similar in this particular experiment It is difficult to test a fibrinolysin for traces of thrombin because the enzyme lyses the test fibrinogen before it has a chance to clot with a very weak thrombin We did perform this control test however in the presence of antiproteases (esp antifibrinolysin) which seemed to rule out this possibility It is not possible therefore to deduce a common mode of action for these two dissimilar agents (thrombin and fibrinolysin) with any factual knowledge currently available

Conclusion We present the experimental facts therefore which *prima facie* indicate that (1) platelets have a weak thromboplastic action in systems such as those described and that (2) weak thrombin and (3) serum fibrinolysin each have a significant 'potentiating' action on this platelet effect Experiment 37 was even more satisfactory and controlled than the recent confirmatory Experiment 36

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Preliminary Studies These will be cited in later sections These clearly established the following facts needed for interpretation of the thrombin forming experiments

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II In higher concentrations than those selected for the present experiments trypsin was thrombinolytic and Loomis' fibrinolysin very feebly so requiring several days incubation in order to demonstrate this

III Again in too high enzyme concentrations to be significant in the present experiments a question of prothrombinolysis might arise

430 301 However Dr Lewis Ann C Howe and the author [299]

could not confirm this but found instead a marked susceptibility of AcG to the proteolytic actions of fibrinolytic enzymes

IV When thrombin formation is very slow and weak it is possible to encounter fibrinogenolysis by the enzymes before the weak thrombin has a chance to form a clot. In fact this is indeed one of the author's [137] methods for assaying trypsin and other fibrinolytic proteases at high dilutions as will be illustrated in Experiments 44 and 45 pp 117 and 118 respectively

V There are quantitative relationships between enzymes and the respective enzyme inhibitors which must be worked out and observed [291 292]. Furthermore the enzyme inhibitors act in different ways [292] and some of them require a time period hence pre incubation with the enzyme in order to exert significant antiprotease effects. This pre incubation is indicated by an asterisk (*) with designation of the anti enzyme used

All these considerations were carefully tested before using the previously described (pp 111 112) reagents in the following groups of tests

Experiment 38 (1948) Table XLI

Purpose To test antiproteases on platelet and trypsin additives in the thrombin forming system

Reagents Plat 0.1 ml conc. suspension (p 112) Tryp : 0.01% Kunitz's crystalline trypsin (p 111) 0.3 ml P.I. 0.1% pancreatic inhibitor (p 112) 0.1 ml Afln 4% Loomis' anti fibrinolysin (p 111) 0.3 ml Pro 0.1% Seegers prothrombin (p 111) 0.2 ml AcG 0.1% Ware and Seegers' serum AcG (p 111) 0.2 ml Ca 0.5 ml 0.05M CaCl₂

Method and Results These are shown in Table XLI. They demonstrate (1) weak thromboplastic action of platelets; (2) similarly weak thromboplastic action of trypsin alone; (3) marked potentiation of thromboplastic action when trypsin is added with platelets; (4) inhibition of the last by P.I. and (5) similar inhibition by Afln*

Clot lysis This was followed over 3 weeks in these experiments. It was noted in 3 days in (2) but was completely inhibited by the platelets (3) as well as by the antiproteases (4 5)

Experiment 39 (1948) Table XLII

Purpose To test antiproteases on platelet + fibrinolysin additives in the thrombin forming system

Reagents Same as in Experiment 38 except for (a) use of 0.4 ml 0.5% Loomis fibrinolysin (p 111) instead of trypsin and (b) additional test with 0.2 ml 0.01% S.B.I. (soybean inhibitor p 112)

Method and Results These are shown in Table XLII

Discussion Test (1) of Experiment 38 (Table XLI) was also the control with platelets alone for the additional tests in Experiment 39

(Table XLII) (6) the fibrinolysin alone had a very weak thromboplastic effect as compared with the control (11) in which Ca and AcG alone caused no detectable thrombin in tests watched for ½ hr (7) the fibrinolysin however markedly potentiated the platelet thromboplastic action apparently like trypsin did in tests (3) of Table XLII (8) Unlike the trypsin experiment (4) however P I did not inhibit this action of fibrinolysin (9) Neither was Afln* able to cause any significant inhibition of the fibrinolysin's potentiating action again differing from the corresponding trypsin experiment (5) S B I (10) seemed to inhibit in the earlier phases although much less so later It will be shown (p 116) that this can be explained as due to a direct anti thromboplastic action of the S B I itself In fact it is some what significant that this was eventually largely overcome by the fibrinolysin preparation

Experiment 40 (1956) Table XLIII

Purpose To test antiproteases in thrombin forming systems in which the thromboplastic additives are (I) platelets only (II) platelets + antihemophilic globulin

Reagents Eluate dialyzed (p 81); to provide prothrombin proconvertin PTC etc (0.2 ml per 5 ml T M)

Plat 0.2 ml of a new preparation of human platelets (normal) p 54

AHG: Dr R H Wagner's antihemophilic globulin preparation described on p 77 (0.2 ml)

S.B.I.: 0.1 ml of a new 0.1 M borate buffer solution of crystalline soybean inhibitor (Worthington Biochemical Lab Freehold N J)

P.I. Dr L A Kazal's pancreatic inhibitor preparation (p 112) 0.1 ml of 1% sol in borate buffer

N.B.I.*: Dr D E Bowman's re purified navybean inhibitor preparation (p 117) 0.1 ml of 0.1% sol in bor buffer

Afln*: Dr E C Loomis 1947 preparation (p 111) A 0.4% two day old extract (freed from insoluble material) in bor buff 0.3 ml The last two inhibitors* were pre incubated for 15 min at 28°C with the thromboplastic additives (I) or (II) respectively

T.M.s: Made up to 4.5 ml with borate buffer and then activated with 0.5 ml 0.04M CaCl₂

Results These are given in Table XLIII They reveal:

I (1) Platelets alone were significant activators

(2) S B I was markedly inhibitory (anti thromboplastic) especially in the earlier phases of activation

(3) P I and (4) N B I * were not inhibitory A very minor improvement was hardly significant

(5) Afln* was distinctly inhibitory but whether this was due to the very old preparation could not be determined as the last of this material was used up in this test

II (6) AHG caused the same important potentiation (? thromboplastin generation) of platelets as with cephalin (see test 7 Table XIII) Compare with test (1)

could not confirm this but found instead a marked susceptibility of AcG to the proteolytic actions of fibrinolytic enzymes

IV When thrombin formation is very slow and weak it is possible to encounter fibrinogenolysis by the enzymes before the weak thrombin has a chance to form a clot. In fact this is indeed one of the author's [137] methods for assaying trypsin and other fibrinolytic proteases at high dilutions as will be illustrated in Experiments 44 and 45 pp 117 and 118 respectively

V There are quantitative relationships between enzymes and the respective enzyme inhibitors which must be worked out and observed [291-292]. Furthermore the enzyme inhibitors act in different ways [292] and some of them require a time period hence pre incubation with the enzyme in order to exert significant antiprotease effects. This pre incubation is indicated by an asterisk (*) with designation of the anti enzyme used

All these considerations were carefully tested before using the previously described (pp 111-112) reagents in the following groups of tests

Experiment 38 (1948) Table XLI

Purpose To test antiproteases on platelet and trypsin additives in the thrombin forming system

Reagents Plat 0.1 ml conc suspension (p 112) Tryp 0.01% Kunitz's crystalline trypsin (p 111) 0.3 ml P.I. 0.1% pancreatic inhibitor (p 112) 0.1 ml Afln 4" Loomis' anti fibrinolysin (p 111) 0.3 ml Pro 0.1% Seegers' prothrombin (p 111) 0.2 ml AcG 0.1% Ware and Seegers' serum AcG (p 111) 0.2 ml Ca 0.5 ml 0.05M CaCl_2

Method and Results These are shown in Table XLI. They demonstrate (1) weak thromboplastic action of platelets; (2) similarly weak thromboplastic action of trypsin alone; (3) marked potentiation of thromboplastic action when trypsin is added with platelets; (4) inhibition of the last by P.I.; and (5) similar inhibition by Afln*

Clot lysis This was followed over 3 weeks in these experiments. It was noted in 3 days in (2) but was completely inhibited by the platelets (3) as well as by the antiproteases (4; 5)

Experiment 39 (1948) Table XLII

Purpose To test antiproteases on platelet + fibrinolysin additives in the thrombin forming system

Reagents Same as in Experiment 38 except for (a) use of 0.4 ml 0.5% Loomis fibrinolysin (p 111) instead of trypsin and (b) additional test with 0.2 ml 0.01% S.B.I. (soybean inhibitor p 112)

Method and Results These are shown in Table XLII

Discussion Test (1) of Experiment 38 (Table XLI) was also the control with platelets alone for the additional tests in Experiment 39

future. Qualitatively at least platelets seemed to be potentiated by trypsin in very much the same way as cephalin [149] (p 105) and weak thromboplastin [156] (pp 105 106) were. We concluded that there was an important common denominator in these comparisons. It could be a common thromboplastic phospholipid mobilized or made available to an enhanced degree by the disaggregating (Pope [379] cited [138]) action of the proteolytic enzyme. Such an idea fits the author's cephalin availability theory and could explain the significance of thromboplastic enzymes' like trypsin (cf p 104).

(7 C) CONTROL TESTING OF ENZYMES AND ANTIPROTEASES

Experiment 43 (1948) Table XLVI

Purpose To show that anti proteases have no significant effect on the thrombin fibrinogen reaction

Method Timing of the clotting of a test fibrinogen with pre incubates of thrombin and the various antiprotease preparations. 2 ml thrombin (bovine Parke Davis & Co s thrombin topical p 56 5 units/ml) + 2 ml antiprotease (or borate buff in control) incubated for periods of $\frac{1}{4}$ min to 1 hr and 0.5 ml samples of mixture tested on 0.5 ml B.F. Armour's (1st) p 55

Results These are given in Table XLVI. They were negative with regard to any significant effects of (1) P.I. (p 112) 0.1% (2) S.B.I. (p 112) 0.005% (3) N.B.I. (below Experiment 44) 0.01% (4) Afln (p 111) 0.1% even with an hour's pre incubation with the thrombin

Experiment 44 (1956) Table XLVII(A)

Purpose To demonstrate proteolytic activity in a long preserved (>11 yr.) trypsin preparation and to show its inhibition by two types of trypsin inhibitors

Method The fibrinogenolytic technique originally described by the author [137] in 1943

Reagents Borate buffer pH 7.7 (p 111) Thr bovine thrombin (Upjohn's p 56) 20 units/ml B.F. 1st bovine fibrinogen (Armour's BaSO₄ treated p 55)

Tryp The >11 yr. old (commercial) trypsin preparation used in Experiment 35 (p 108). The 2nd stock solution was diluted 100x with borate buff

N.F.I. a highly purified (reprecipitated with (NH₄)₂SO₄) antiprotease from navy beans prepared by Dr D. E. Bowman [59] and kindly supplied us in 1948. A fresh 0.1% solution of the dried preparation was made up in borate buff

P.I. A highly purified pancreatic trypsin inhibitor prepared January 2, 1948 (Lot No. P.A. 97.1) by Dr L. A. Fazal (Sharp and Dohme Labs. Glenolden Pa.) Fresh 0.1% solution in borate buff

L.M. (lysing mixture) 1 ml B.F. + 0.5 ml trypt + 0.5 ml inhibitor incubated at 28°C with testing of 0.2 ml samples at intervals on adding

(7) S B I was very antithromboplastic in this mixture also (cf (2))

(8) P I and (9) N B I* were non inhibitory

Experiment 41 (1948) Table XLIV

Purpose To test antiproteases in thrombin forming systems activated by Ca and tissue thromboplastin

Reagents and Method Same as in Experiments 38 and 39 except for use of 0.5 ml ipln. A (p 90) as the only thromboplastic additive

Results These are given in Table XLIV They show:

(1) Marked activation with this strong tissue thromboplastin

(2) Its complete inhibition with S B I an important control finding (cf Experiment 39)

(3) No inhibition by P I and (4) none by Afln *

Experiment 42 (1956) Table XLV

Purpose To test antiproteases in thrombin forming systems activated by Ca and cephalin

Reagents and Method Same as Experiment 40 (I) namely using dog eluate (prothrombin etc) AcG borate buffer but substituting 0.1 ml 0.1% cephalin (p 71) for the platelets

Results These are given in Table XLV They reveal

(1) Satisfactory activation by the cephalin

(2) S B I (p 112 0.1 ml of 0.1% solution) was again anti thromboplastic (cf tests (2) and (7) Table XLIII and test (2) Table XLIV)

(3) P I (p 112 0.1 ml of 0.1% solution) was not inhibitory neither was (4) N B I* (p 117 0.1 ml of 0.1% solution)

General Discussion of Experiments 38-42 Of the various antiproteases tested the soybean inhibitor introduced complications because of its direct anti thromboplastic effects against platelets (Experiment 40 (I)) or platelets + AHG (Experiment 40 (II)) tissue thromboplastin (Experiment 41) or cephalin (Experiment 42) The others were reliable inhibitors of the enzymes trypsin and fibrinolysin in the systems studied and had no significant antithromboplastic effects of their own Antifibrinolysin in Experiment 40 (I) was a questionable exception Inhibition by antiproteases of the platelet thromboplastin potentiating action of trypsin was good evidence that this phenomenon depended upon the proteolytic actions of the pancreatic enzyme On the contrary the apparently similar potentiating (to platelet thromboplastin) effects of fibrinolysin were not removed by antiproteases in amounts sufficient to suppress all proteolytic action This unexpected difference could not be explained except by postulating some unknown factor in the enzyme preparations used A suggestion that it might be the 'Hageman factor' [401] was noted (p 26) in the introductory section but further exploration of this must await the

excellent degree of activity and was judged suitable for the tests with platelets in Experiment 36. The antifibrinolysin was also satisfactory. Similar tests were made with the enzyme systems used in the 1947-49 experiments previously cited.

(7 D) FIBRINOLYTIC PHENOMENA AND PLATELETS

The use of borate buffer with its mild bacteriostatic effect proved very valuable as a preservative in our earlier [156] prothrombin activation experiments and enzyme studies (ref [291]). In these systems it was often possible to demonstrate weak fibrinolytic enzymes requiring days or weeks to produce clot lysis. We shall cite only those incidental observations which pointed to certain relationships of the platelets to proteolytic phenomena. Chief among these was the demonstration of an antiprotease in platelets particularly noted in the inhibition or retardation of fibrinolysis by trypsin. This is evident in the clot lysis data of Table XLI (Experiment 38 p 114). References to platelet anti fibrinolysin are given on p 51. We had observed it in Experiment 39 and other similar experiments in 1947-49.

(7 E) THE 'AcG LIKE' FACTOR IN PLATELETS [476]

Experiment 46 (1950) Table XLVIII

Purpose To test for an accelerator factor in washed platelets using an AcG poor prothrombin activated by Ca and a strong tissue thromboplastin. Comparison with varying concentrations of Ware & Seegers' AcG preparation.

Method The usual two stage technique (1) without added AcG (2) with platelets and (3) and (4) with varying concentrations of added AcG.

Reagents Pro One of Dr Seegers' highly purified bovine prothrombins Tpin. B; Soluplastin¹ (p 56) Plat p 112 AcG p 111 T.M.'s as described in Table XLVIII.

Results These are given in Table XLVIII.

Discussion The poor thrombin formation in (1) with tpin only indicated the accelerator deficiency in the chosen prothrombin. In (2) platelets were found to have only a little effect. Since there was already a strong thromboplastin present, this small effect could have been due to some 'AcG like' factor in the platelet preparation. Comparisons with the two additions of 'purified serum type AcG' (3, 4) suggested that this platelet factor was roughly comparable to what amounted to a concentration of only 1:2 500 000 in the final thrombic mixture or 1 100 000 equivalent vols of platelets and AcG. Questions as to the lability of 'platelet AcG' during the manipulations of platelet preparation were difficult to answer. We have found significant amounts of platelet AcG in our more recent (1952-56) clinical routine tests discussed later (see Table LII).

to 0.2 ml Thr Successive prolongations of the clotting time indicate the rate and extent of the fibrinogenolysis

Note The P I acts immediately and is added just before the trypsin. The N B I* on the other hand resembles serum fibrinolysin in requiring a period of time to inhibit the proteolytic enzyme [292]. Hence 20 min pre incubation of N B I with trypsin was allowed before adding the fibrinogen. Borate buff was used to make up the 2 ml L M vol as needed

Incubates

- 1) Control without enzymes
- 2) Trypsin alone;
- 3) Tryp + N B I*
- 4) Tryp + P I

Results These are given in Table XLVII(A). They clearly show:

- 1) stability of fibrinogen in buffer (control)
- 2) marked fibrinogenolysis by the diluted old trypsin;
- 3) complete inhibition of lysis by the N B I*;
- 4) almost complete inhibition also by the P I

Conclusions This experiment affords evidence of the remarkable stability of trypsin at ordinary refrigerator temperatures (4°C) in glycerol borate buffer (equal vols) solution. It indicates suitability of this old enzyme preparation for the special platelet potentiation tests of Experiment 35 (p 108) and anti heparin Experiment 47 (p 120). Similar tests were made on this preparation and on crystal line trypsin solutions preliminary to the 1947-1949 experiments (p 111 et seq).

Experiment 45 (1956) Table XLVII(B)

Purpose To test the proteolytic activity of an old (>43 months) dog serum fibrinolysin preparation and its inhibition by antiproteases

Method The fibrinogenolytic technique [137] as used in Experiment 44 also employing the same reagents except for: (a) replacing the trypsin by lysin a purified fibrinolytic enzyme (plasmin or trypase) from dog serum prepared by Dr. Jessica H. Lewis and the author [291] October 29, 1952. A 1:40 dilution in borate buffer had been preserved frozen at 20°C. (b) Afln (0.4% in borate buff) was prepared by 2 day extraction at 4°C of a November 1947 dried anti enzyme preparation of Dr. E. C. Loomis (see p 111).

Data These are shown in Table XLVII(B). They demonstrate:

- 1) Stability of the control fibrinogen (buffer only)
- 2) Marked fibrinogenolysis by the old enzyme solution complete in 10 min
- 3) Inhibition of proteolysis when the enzyme was pre incubated for 15 min with 0.1% N B I* (equal 0.5 ml vols)
- 4) Similar inhibition by Afln* (also pre incubated 15 min with the lysin)

Conclusion This dilute enzyme preparation also preserved an

hence restore the platelet (+ enzyme) thromboplastic activity was very strikingly demonstrated by these tests

(5) The trypsin alone was only weakly thromboplastic. This strongly suggested that the activity which was released from the heparin inhibition by trypsin was not that of the enzyme itself but must have come from the platelets

(6) The strong tissue thromboplastin (Soluplastin) was also able to overcome the inhibitory effects of heparin in this thrombin forming system. This again confirms our 1939 Experiment (29 p 100 tests 5 6)

Discussion Unlike our earlier experiments (29 31) which used excessive amounts of heparin as commented upon on p 104 the present study (Experiment 47) employed concentrations of heparin which might be compared favorably with those in the therapeutic or physiological range. Hence it is particularly convincing that such amounts of heparin were able so completely to inhibit the thromboplastic action of platelets in our suitably chosen thrombin forming system. Inability of this heparin however to antagonize tissue thromboplastin or trypsin + platelets was equally significant evidence of the much greater potency of these thromboplastins

Conclusions The foregoing data not only suggest (1) that the usual anticoagulant effects of heparin are intimately associated with inhibition of the thromboplastic function of platelets but also (2) that platelets are much more akin to a simple phosphatide (cephalin) as in Experiment 29 than to ordinary preparations of tissue thromboplastic extracts. Quantitative interrelationships undoubtedly exist and an extremely weak tissue thromboplastin would closely resemble platelets and cephalin in these experiments. However we do believe the facts line up behind the postulates of our 'cephalin availability theory' (See p 143)

(7 G) ANALYTICAL FRACTIONATION OF PLATELETS

This line of investigation was not pursued in this thesis. The reader may be referred to current work in this field by Dr W H Seegers of Wayne University Detroit; but the brief reference we have given to this on p 49 will have to suffice. The one group of analytical data with which the present writer had some connection was that of Mrs Betty Nims Erickson and collaborators in the 1940's cited on p 44. From these we wish to reiterate the important finding (also credited to Chargaff [78] on p 44) that acetone insoluble phospholipid or cephalin is a major fraction of the platelet lipids

(7 H) QUESTION OF A 'FIBRINOPLASTIC FACTOR IN PLATELETS

The author's [134] term 'fibrinoplastic' (see p 17) for factors which accelerate the thrombin fibrinogen reaction through some non specific colloidal or surface effect would seem to be appropriate for such action of platelets (7 fractions) as that claimed by Ware Fahey & Seegers [490] and allegedly confirmed by others (see p 51)

Conclusion These early experiments led us [476] (see p 50) to wonder whether the 'AcG like' factor described by Ware Fahey & Seegers [490] was a genuine platelet component or might merely be adsorbed onto the platelets from the plasma [222]. Our tests which will not be cited in detail indicated the platelet accelerator to be of the plasma AcG type or proaccelerin (see p 26). More about platelet analyses for this factor will be given later in data on clinical cases (p 130).

(7 F) EFFECTS OF HEPARIN ON THROMBIN-FORMATION BY
(I) PLATELETS; (II) PLATELETS + TRYPSIN; OR
(III) TISSUE THROMBOPLASTIN

Experiment 47 (1956) Table XLIX

Purpose To study heparin inhibition of the thromboplastic action of (I) platelets and differences from (II) platelets + trypsin and (III) tissue thromboplastin

Method Essentially the usual method III two-stage technique of following thrombin formation with slight modifications of volumes to control for the heparin carried over into the thrombin fibrinogen (clotting test) mixtures

Reagents Eluate (dialyzed No 127) p 75 was used to provide prothrombin proconvertin etc 0.2 ml/5 ml T M

Plat Human platelets (p 54) 0.2 ml

Tryp : 0.5 ml of a fresh 1:100 dil with imid buff sol (pH 7.3) of the 11 yr old stock (2%) trypsin described on p 108

Tpln : 0.2 ml 'Soluplastin' (p 56)

Ca 0.04M CaCl_2

Hep : imid buff sol dilutions of heparin ('Liquaemin Organon Inc (Hoffman LaRoche) Orange N J) 0.5 ml of 2 units/ml hep in T M (b series); 0.1 ml of 0.2 units/ml hep in (c) series of fibr + T M tests

T.M.'s and clotting test mixtures (0.2 ml fibrinogen (dog) + 0.1 ml (a) 0.2 units/ml hep or imid buff sol + 0.1 ml T M); are described in the caption of Table XLIX

Results (Table XLIX) show in:

(2) practically complete inhibition of platelet thromboplastic activity by heparin in concentration of 0.2 units/ml in the thrombin forming mixture

(1 a c) was the appropriate control supplying the same final concentration of heparin namely $2/4$ of $0.2 = 0.1$ unit/ml as in (2) tests

(1 a d) with no heparin were only a little better than (1 a c) thus showing that the effects of heparin in the concentrations tested were very minor on the thrombin fibrinogen reaction

(3) the significant trypsin potentiation of the thromboplastic action of platelets again confirms the data of Experiments 35-38. The only difference was that in Experiment 47 the final clotting tests were performed in the presence of heparin

(4) The ability of trypsin to overcome the heparin inhibition and

original (0) value. The rapid drop in the platelet counts thereafter probably represents chiefly the removal of platelets by entanglement in the fibrin clot. (B) The hemophilic blood started clotting in 25 min. Averaging the 4, 5 and 7 min tests the platelet count had fallen in this time to 76%, only (137.3/182) of the original (0) value. The pre clotting 10-15 min period i.e. 3 observations gave a similar value viz 87% within the 'experimental error'. Only after clotting was there an abrupt fall in the platelet count suggesting removal of the formed elements entangled in the fibrin clot.

Conclusion. There seems to be a significant preservation ($80 \pm 4\%$) of platelets during the longer pre clotting period in hemophilic as compared with normal (32%) human blood. This could reflect a diminished 'utilization' of platelets in the pre clotting phase of hemophilic blood. Platelet counts after the onset of clotting are not significantly different and in all probability depend upon platelet removal by entanglement in the fibrin clot.

8 DARK FIELD MICROSCOPIC OBSERVATIONS OF PHOSPHOLIPIDS PLATELETS AND OTHER FORMED ELEMENTS OF THE BLOOD AND BONE MARROW

(8 A) FOREWORD

Siedentopf & Zsigmondy [441] applying the optical principles worked out by Abbe and Helmholtz first used the dark field method of microscopy. It was quickly applied to the study of living cells and micro organisms by numerous investigators in Germany, France and elsewhere. The early history of its use for study of the blood was reviewed in 1913 by Aynaud and Jeantet [27]. Aynaud [24, 25, 26] used it as well as ordinary microscopy in a monumental study of the alterations of the blood platelets in a work which deserves far more recognition than it has received by occasional brief mention in the more modern reviews [411-415]. Stubel [459] in 1914 gave some good descriptions of the dark field appearances of platelets with excellent photomicrographs. The present author demonstrated cellular and clotting appearances in a drop of human blood preserved at body temperature under the dark field microscope before the British Association for the Advancement of Science meeting at Cape Town in 1929. This initiated an interest pursued from time to time over many years leading to publications in South Africa [122] and in America [124-139]. The following account summarizes those observations relating to the role of phospholipids in the alterations of platelets and other formed elements which occur after withdrawal from the body. It is original more particularly in the novel explanation which is arrived at from an analysis of the data reviewed.

(8 B) PLATELETS

1 Alteration during blood coagulation (Figure 18)

Method. A simple small drop of finger prick blood is placed on a scrupulously clean slide and spread thinly with gentle pressure from

B L Travis and the present author in 1947-49 (p 115 of ref [476]) were unable to demonstrate any significant effect of (dog) platelets on the thrombin fibrinogen reaction

Experiment 48 (1956) Table L

Purpose To re study the effects of adding platelets to thrombin fibrinogen mixtures

Reagents Plat 0.1 ml of normal human platelet suspension (pp 54-112) Thr bovine thrombin (Upjohn's p 56) 0.1 ml of serial dilutions with imid buff saline (p 56) of 20 unit/ml stock solution Fibr 0.2 ml of (dog) BaSO₄ treated fibrinogen (p 55)

Method Clotting time (sec) at 26°C of thr + plat (or buff saline in controls) + fibr

Results are given in Table L. They show no significant effects of the platelets (1) as compared with the controls (2)

Discussion The tests were meticulously performed. The duplication of values in repetition of the last two measurements indicates the insignificant experimental error. Essentially similar results were obtained with the 3½ yr old dog platelet preparation described on p 95

Conclusion We are unable to confirm any 'fibrinoplastic' action of platelets and wonder whether such findings by other workers may not be the result of some alternative (? denaturation) phenomena. Denatured proteins often do have some effects on the thrombin-fibrinogen reaction [60]

(7.1) PLATELET UTILIZATION DURING CLOTTING

Experiment 49 (1955) Table LI

Purpose To investigate the rate of disappearance of platelets during the clotting of (A) normal and (B) hemophilic bloods

Method Bloods were collected by clean puncture of the antecubital vein using No. 20 new needle and siliconized syringe. (I) was immediately placed in a siliconized tube at 37°C and samples pipetted off at the stated intervals (Table LI) into a series of siliconized red cell hemocytometry pipettes and the platelet counts [62] made as soon as practicable thereafter. (II) 4.5 ml of blood was mixed with 0.5 ml 0.1M sod citrate in a siliconized pyrex bottle and held at 37°C until the control period when its platelets were counted as usual.

Results are given in Table LI

Data (A) The normal blood clotted soon after removal of the 6 min sample. Averaging the 5 and 6 min tests i.e. just before clotting the platelet count had dropped in this time to $104.5/320 = 32\frac{1}{2}\%$ of the

is far more common for them to remain for hours either anchored to the residue of the platelet body or occasionally breaking away and floating free. The clubs and filaments are essentially similar to the vesicles except in shape and in the lack of particulate contents. They are very delicate and oscillate vigorously with the jerkiness of typical Brownian movement. They frequently become detached. The feebly refractile double contoured free wriggling filaments are characteristic objects. They often adhere to red cells and other objects. We suggest as did Grassberger [472] for the erythrocyte filaments (see p. 128) that a number of observers in the past have mistaken these for spirochaetal (or other) micro organisms and erroneously tried to explain certain diseases (e.g. Hodgkin's disease, rheumatoid conditions etc.) as 'infections' with such agents.

From the body of the platelet we [124] have seen on one occasion during the earlier phases of alteration a single bright particle jerking in and out of the parent element in a manner resembling the agonal movements of the granules in dying leucocyte fragments as described by the author [122] in 1930. This could be regarded as evidence that the platelet is a necrobiotic cellular (see megakaryocyte) fragment. Such outline of the body of the platelet as may remain visible after formation of the 'excrescences' exhibits delicate form changes for a short time but soon ceases to be distinguishable especially when several platelets adhere together. The ultimate result of platelet alteration is the formation of an adherent granular matrix of large and small particles in which body outlines are no longer visible. Adhering to the mass are numerous vesicles, clubs and filaments and often several fibrin needles (see Figure 1 of [124]).

2 Agglutination of platelets.

This characteristic phenomenon of normal platelet alteration was first described by Hayem [213-214] in 1877-1878 and especially studied in 1917 by J. H. Wright and G. R. Minot [11] under the name 'viscous metamorphosis' coined by Eberth & Schimmelbusch [113] in 1886.

Under the dark field microscope it is seen that the platelets which happen to be thrown together while acquiring the stickiness previously noted during the period of swelling readily adhere to form a clump which is increased by the addition of other platelets that chance along. It is this removal by agglutination and adherence to glass and other wettable surfaces rather than disintegration which makes it so difficult to find platelets in serum from clotted blood and also renders any attempt at counting of the platelets invalid if even a trace of clotting has occurred. Aynaud [24] and others including ourselves [124] used paraffined surfaces to minimize these clumping phenomena. The more recent introduction of non wettable siliconed surfaces is an improvement over these earlier methods which we have frequently used in more recent years and which has been commented on in some detail by Marjorie B. Zucker [518] as noted on p. 38. The phenomena of platelet clumping and partial disintegration (or 'alteration') normally in vitro make it difficult to study specific platelet agglutination and platelet lysis e.g. after sensitization to certain drugs or antiplatelet sera [1]. Such studies must be performed with extremely careful technique and the use of siliconed surfaces.

a No 0 coverslip. It is examined under the oil immersion lens of a good dark field microscope with an adjustable diaphragm in the objective to permit best optical resolution of the minute details observed. A bright tungsten band or similar evenly focussed (e.g. Burton lamp) illuminant is adjusted for optimal results and the cardoid condenser (dark field) is accurately centered. The heat of the lamp usually warms up the blood drop sufficiently without requiring the 37°C chamber (enclosing the whole microscope) which was used in our earlier observations.

Observations. There are no significant alterations in the chylomicrons ('hemoconia') erythrocytes and leucocytes [124] in the few minutes which elapse before the appearance of the fibrin 'needles' which indicate the clotting of the blood. The platelets, however typically 'alter' so rapidly that it is rare to observe them in their normal circulating form namely that of a tiny ovoid disc about $\frac{1}{4}$ - $\frac{1}{3}$ the diameter of an erythrocyte and with a delicate feebly refractile contour a clear translucent periphery (optisch leer Stübel [459]) and a few central granules of varying refractility which are devoid of Brownian or other movement. There is no trace of any nucleus. Viewed in profile the platelet appears 'kennetjle' ('tip cat' or 'batonnet' Aynaud [24]) shaped or fusiform ('spindelförmig' Stübel [459]) see p. 35. Altered forms of platelets occur in a matter of seconds after withdrawal of the blood. First there is a swelling into a spherule the shape of which is quite evident when it happens to be rolling over in fluid currents in the plasma. This form has an even fainter outline but more distinct inner granulation consisting of the larger and brighter particles formerly observed together with a background ('granulomere') of finer hazy granules only faintly visible. It attains to three or four times the size of the original platelet and evinces a 'stickiness' which causes it to adhere to the slide or coverslip. Once adherent its outline may continue to expand apparently from several foci until a diameter of some 10 μ is reached. We term this a spreader form. It is seen in only a small minority of the altering platelets however. Much of the commonest change is that which has been frequently described by earlier observers namely the stellate form (see Plate I of Figure 18). This shows a number of processes or as we prefer to call them 'excrescences' protruded from the margin of the platelet and taking up practically all the material of the outer layer or 'hyalomere'. The excrescences are of three kinds viz (1) rounded or 'vesicular' ('bosselures' Aynaud 'kugelige Bläschen' Stübel) which are most frequent under these simple conditions (see Plate 3 of Figure 18) (2) club like and (3) filamentous (see Figure 3 in [124]).

The delicate vesicles of (1) above seem to hover like a captive balloon at the surface of the platelet. After a certain degree of swelling they have been observed to shrink temporarily with an adjacent eddying of hemoconia (chylomicrons) suggesting release of contents into the surrounding plasma. As they gradually swell again there appear a few granules some apparently entering in from the body of the platelet and these granules show vigorous Brownian movement suggesting that the contents of the vesicle have now become liquified. Several vesicles usually form from each platelet and their size varies up to 5 or 6 μ . Sometimes they rupture completely and disappear but it

is far more common for them to remain for hours either anchored to the residue of the platelet body or occasionally breaking away and floating free. The clubs and filaments are essentially similar to the vesicles except in shape and in the lack of particulate contents. They are very delicate and oscillate vigorously with the jerkiness of typical Brownian movement. They frequently become detached. The feebly refractile double contoured free wriggling filaments are characteristic objects. They often adhere to red cells and other objects. We suggest as did Grauszberger [472] for the erythrocyte filaments (see p. 128) that a number of observers in the past have mistaken these for spirochaetal (or other) micro organisms and erroneously tried to explain certain diseases (e.g. Hodgkin's disease, rheumatoid conditions, etc.) as 'infections' with such agents.

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3 Platelets and fibrin

The often reported finding of fibrin needles or filaments adhering to altering platelets is in fact less important than the observations: (1) platelet alterations commence long before fibrin appears (2) many fibrin needles can be seen under the dark field to appear de novo out of the background of the clear plasma by no means requiring the platelets (or other visible object) as foci for their formation

4 Platelet preservation

The mere use of a non wettable surface e.g. silicone sans anti coagulant has only a minor effect in delaying the typical platelet alterations which soon show all the appearances which we have described. Of course it is highly probable that complete maintenance of non wetability is impossible under the conditions of observation and that there is bound to be some air liquid interface

Anticoagulants afford a much more practical means of controlling platelet alterations. Aynaud's studies see p 123 must be given full credit for the thorough investigation of very many added agents investigated for their effects on platelet alterations. We have merely used citrate oxalate and more recently sequestrene- Na_2 (EDTA see p 79) to confirm the ability of the decalcifying anticoagulants to preserve platelets. By preservation we mean that the alterations which do still occur are much less disintegrative. All changes are greatly retarded and there are some definite differences from the sequence of events which we have described above. Agglutination of the platelets is particularly delayed and may be negligible even after days especially with sequestrene combined with the use of siliconed surfaces.

When citrated platelet rich plasma is placed on an ordinary glass slide for dark field observation many single platelets are observed floating in the plasma. Some however sediment and adhere to an extent sufficient to resist the small fluid currents always seen in these wet preparations. Both free floating and adherent platelets are rather more refractile than in the simple blood drop. The tiny disc form frequently well seen edgewise preponderates at first and may be regarded as the normal form. Its outline is well defined and there is a clear hyalomere and a few central stationary granules (granulomere). It soon begins to show delicate short wavy filamentous excrescences from one or more points along the edge. Confirming Stübel [459] we have sometimes seen these filaments form withdraw and reform but they soon remain extended and irreversible. After a few minutes they appear to become more definitely rigid. They seem to extend further over the course of several hours and may reduce the body of the platelets to insignificance. Sometimes a single very long filament extends from the small granular remnant of the body in what we have termed a sperm like form [124]. A number of rounded excrescences usually one (or two) to a platelet may also be observed. Unlike the vesicles of unpreserved platelets however these can be seen edgewise to be disc like. Hence we [124] use the term plaque form. Except with vigorous artificial manipulation it is unusual for either the filiform or plate-like excrescences to become detached. Preserved platelets may be washed with saline (0.85% NaCl) in silicone and kept in the frozen stage (-20°C).

Recalcification results in a re acceleration of the retarded alterative phenomena in the platelets and if the plasma is still present fibrin formation occurs a few minutes later. The recalcified platelets swell, adhere, clump and (partially) disintegrate in much the same manner as in unpreserved blood and the phenomena are essentially similar in recent preparations and in long preserved platelets (in silicone at 40°C) recalcified as usual. See Plate 2 of Figure 18. It is rather remarkable to see the rigid excrescences soften and swell and vesiculate, often involving the whole of the platelet except for most of the granulomere. Granules evincing Brownian movement may appear in the vesiculated forms.

Effects of varying citrate concentrations were minutely studied in our 1934 publication [124] to which reference may be made for details. The important conclusion was that the concentrations of decalcifying agent which prevented clotting also 'preserved' the platelets to the extent of maintaining rigidity of excrescences, lessened clumping and absence of 'fluidity' of platelet content. We regard this as significant evidence that calcium has a specific role in the special ('liquefactive') disintegration phenomena by which the platelets come to play a very important role in the blood clotting phenomena. Osmotic imbibition of water by platelets (1) in hypotonic solution e.g. on diluting with distilled water or (2) on adding saponin (see Plate 4 of Figure 18) in the presence of citrate do not result in plasma clotting.

The conclusion we [124] reached in 1934 was that the platelet 'alterations' consist fundamentally of a 'myelin figure' formation from their surface phospholipids (see later) and that calcium by its influence on the phase relations of the lipid-water system specifically directs the water into the fatty platelet material thereby swelling and disrupting it. This appears to be a significant contribution ordinarily to the platelet release of factors which the platelets contribute to the blood clotting system.

(8 C) MEGAKARYOCYTES [139]

Bone marrow freshly obtained from the femur or tibia of anesthetized rabbits was quickly mounted in autologous serum previously obtained from heart puncture blood of the same animal. Dark field microscopic appearances are shown in Plates 5 and 6 of Figure 19.

In the former, no anticoagulant was used and vesicular excrescences containing granules oscillating with Brownian movement are seen at the periphery of the megakaryocyte (in a small proportion of the giant cells observed). The excrescences are very similar to ordinary platelet alterations and like the latter often become detached. In the second series of observations the marrow preparation was citrated (Plate 6). This suppresses the slow amoeboid movement of the megakaryocytes and causes them to show a well defined outline and a fixed glassy appearance of their very granular cytoplasm (very similar to leukocyte appearances in citrated blood preparations). A very few vesicles (see Plate 6) continue to appear however but are smaller, more refractile, devoid (or almost) of granules and resemble the plaque form of correspondingly treated platelets (p. 126).

We have never observed a living megakaryocyte fragmenting into platelets [510] and believe the phenomena described are absent or less

marked in the less mature megakaryocytes. They were not observed in polykaryocytes (osteoblasts) which are unrelated to platelet origin

(8 D) THROMBOCYTES [139]

The nucleated thrombocytes of amphibia (frog) reptiles (turtle) fish (fresh water sun fish) and birds (fowl) have been studied under similar conditions. Plate 7 (Figure 19) shows an altered frog thrombocyte lying on top of a large fibrin filament. Note the vesicular bodies derived from the thrombocyte some still attached and others released for some distance. As frequently noted in the earlier literature on thrombocytes their disintegration is often very sudden and explosive in character. This scatters excrescences chiefly vesicles and granular material to some distance from the parent thrombocyte as well shown in Plate 8 (Figure 19) in the case of the sun fish observation. It is noteworthy however that many vesicles persist in the region of the original adherence and disintegration of the thrombocyte. The dark field observations confirm the essential similarity of the alterations of thrombocytes megakaryocyte margins and mammalian platelets.

(8 E) ERYTHROCYTE STROMATOLYSIS ETC

Furchgott [182] in 1940 made dark field observations of altering erythrocyte ghosts (stroma of hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate. He was apparently unaware of our earlier data on platelet alterations and independently suggested the 'myelin figure' explanation of the phenomena observed. His search of the red cell literature revealed several earlier observations (e.g. Oliver [355] Kite [267] Auer [23] and others) of essentially similar appearances from both hemolyzed and non hemolyzed red cells. In some observations made by the present author in Cape Town in 1930 and recorded in the photomicrograph reproduced in Plate 9 Figure 20 'stromatolytic' filaments were observed extending from the margins of young erythroblasts in a drop of heart blood from a rabbit embryo. Plate 11 Figure 20 photographed by an erstwhile graduate student (P. H. Ralph) working under the author's supervision at the University of Michigan in the early 1940's shows apparently similar thread like processes connecting points at which normal adult human red cells have apparently touched and then withdrawn from one another. Plate 10 Figure 20 also prepared by Dr. P. H. Ralph is a striking example of the 'spiculated stromatolytic' appearances occurring in vitro in slide (dark field microscopy) observations of a nucleated red cell from a tadpole [139].

Conclusion. There is clearly a common phenomenon connected with surface alterations of the several varieties of blood and bone marrow formed elements collectively considered in the foregoing observations. The basis for attributing these phenomena to a myelin figure formation from the surface phospholipids of these formed elements follows from the next series of observations.

(8 F) MYELIN FIGURE FORMATIONS FROM PHOSPHOLIPIDS

R Virchow [483] first described myelin forms (figur. 3 as microscopical appearances at the edges of nervous tissue observed in contact with watery solutions. He interpreted them in terms of surface (interphase) relations between the lipoid and aqueous phases. J. B. Leathes [284] studied them very extensively in 1925. He photographed at successive $\frac{1}{2}$ minute intervals the large myelin figures appearing at the edge of a film of lecithin in contact with a wide variety of watery solutions chosen to illustrate the effects of (a) specific ions (b) osmotic concentration and (c) adsorbed substances. The myelin forms were readily produced by distilled water. Leathes noted a suppressive or inhibitory effect of calcium ions said to be overcome by 0.01M NaOH and by cholesterol. Other retarding and many favoring agents were recorded by this observer. The present author in 1934 [124] reproduced some of the lecithin experiments of Leathes. Plates 12A and 12B (Figure 20) show the dark field appearance (400x magnif.) of the edge of the phospholipid film: (A) at the start in contact with air showing only the linear highly refractile margin and (B) 3 minutes after contact with distilled water showing the typical large (initial) myelin forms flowing out into the watery phase (upper right part of photo). Clearly visible in the original but poorly reproduced in the photomicrograph (and therefore retouched) is the translucent border as described by Leathes [284].

Our observations established one or two new points which we believe to be of considerable significance.

Firstly all watery solutions cause an initial myelin figure outflow (i.e. into the aqueous phase) which is followed by a further expansion after a 5-10 minute 'delay period'.

Secondly the dark field method permits interpretation of Leathes translucent border as a reciprocal phenomenon namely a penetration of the water into the lipid phase. The secondary expansion phase we observed to be very dependent upon the particular ions in the watery solution. If distilled water or sodium chloride (and similar salt) solutions were used the secondary myelin figure outflow went on with increasing speed and vigor but the inflow of watery phase into the lipid was limited and showed little further penetration beyond that seen in the initial phase. With calcium salt solutions these phase relations were reversed. There was the same initial myelin figure outflow but little extension after the delay period. Secondly however the inflow of this solution into the lipid proceeded apace and without limit extending the 'translucent border' throughout the lipid portion. Our important conclusion was that calcium is specific in directing water into the lipid phase as is well known from the work of physical chemists with phase relations of calcium and sodium salts in soaps and other fat water emulsions.

To Leathes' conclusion that myelin forms are due to surface growth and localized imbibition of water we would add that calcium ions specifically generalize the imbibition and cause a 'reverse' myelin figure appearance as can be seen very clearly in dark field observations of lecithin films.

Applying these ideas to platelet (etc) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc) but in the liquefaction of the excrescences. By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena. What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J Folch (of the Rockefeller Institute New York) which are described on p 98

The chief additional information obtained was that cephalin of the several phospholipids studied yielded myelin forms which in size and appearance e.g. vesicles were often so similar to the above described platelet megakaryocyte and thrombocyte alterations that it seemed reasonable to regard them as identical

Actually the fundamental myelin figure phenomenon was common to most of these phosphatides in contact with watery solutions and calcium salts caused a granular deposition penetrating into the lipid and interfering with its hydrotropic dispersion into the watery phase. The strength of the salt solutions had only minor effects suggestive of some membrane phenomenon in which water penetration rates are partly controlled by osmotic considerations

The inositol phosphatide (III) behaved peculiarly in that it went directly into colloidal suspension as 'chylomicra' instead of showing 'myelin forms'. This is evidently representative of the behavior of the bulk of lipoidal and lipoprotein materials of plasma

Concerning the protein components of the surfaces of platelets etc our experiments have nothing to say

(B G) CONCLUSION

In summary then our comparisons of the dark field microscopical appearances of (a) water phospholipid films and (b) in vitro 'alterations' of platelets megakaryocytes thrombocytes and certain erythrocytes indicates the fundamental importance of myelin forms (figures) and related phenomena at water lipid interfaces. We believe these to be extremely primitive and essentially non vital phenomena of lipid rich surface membranes. Not only is there an hydro tropism of the lipid material causing the appearance of surface 'excrescences' of the various types described but there is also a specific role for the calcium ion. This reverses the phase relationships and directs water into the fatty phase thus contributing to the peculiar disintegrative phenomena studied. Cholesterol proteins osmotic factors pH and perhaps other factors need additional study as further possible modifiers of the basic phenomena which we have described

G CLINICAL CASES WITH PLATELET PROBLEMS

FOREWORD

Our research problem 1950-55 with regard to the investigation of clinical cases with hemorrhagic and related disorders was largely planned and executed by Dr Jessica H Lewis Research Associate

and Co Investigator with advice assistance and some participation by the author. Invaluable technical assistance was rendered by Francis M Morgan Doris C Ferguson Iris Rudin Ann C Howe and Bertha G Jackson. Since 1953 a graduate student Mr James W Fresh has made many able contributions especially to Group I studies (see below) and in the past year Miss Mary Jane Patch has been a most helpful collaborator. The staffs of the University a (North Carolina) Memorial Hospital Duke Hospital the Veterans' Hospital and ORINS Hospital have cooperated with the patient material as have a number of private practitioners. We are indebted to Dr Marjorie B Zucker of the N Y U College of Dentistry Physiology Department for the serum serotonin assays [519] used to assess the platelet vasoconstrictor factor (Item 17 see Tables).

In preparing the data for this thesis the author has proceeded independently and has not consulted Dr Lewis who now has her own Research Laboratory at the University of Pittsburgh. Gratefully acknowledging the leading role of Dr Lewis and the individual contributions of each member of our group the author nevertheless assumes sole personal responsibility for the following presentation and analysis of the data selected.

CASES represent some 86 patients and 32 normal mothers and their infants. They are classified into nine groups. No attempt will be made to analyze them from the clinical viewpoint. Rather they represent merely a physiological contribution of analytical laboratory methods with which to approach a rational diagnosis and understanding of the clinical hemostatic problems. Our groups are chosen from this physiological viewpoint to serve the purpose of this thesis inquiry.

Methods for the most part have been described on pp 53 62 and will not be further elaborated. An itemization (1 30) facilitates organization and reference. In the first Table (LII) the mean and range of test values are given for (A) normal healthy adults (randomized as to sex and age) (B) parturient mothers and (C) their normal newborn infants (cord bloods).

0 \pm (ie trace or minor) + +++ indicate relative degrees of (7) bleeding tendency and (12) clot retraction (in glass). Retraction was also observed in silicone (cf 11) but this will not be reported since the differences from (12) were relatively minor. In group summaries the number of cases was divided for each test into the following categories:

Pos significant alteration of the test value

Neg not significantly different from the normal (norm)

? doubtfully significant

N.T. no test performed

Results are presented in Tables LII LX

GROUP I (A) Normal Adults (B) Parturient Mothers (C) Normal Newborn Infants (Cord Bloods) ref [172] Table LII

(A) Normals were from healthy adults randomized as to sex and age. They were mostly medical students staff and hospital personnel including a few colored helpers. The means of the various tests were

Applying these ideas to platelet (etc.) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc.) but in the liquefaction of the excrescences. By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena. What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J. Folch (of the Rockefeller Institute New York) which are described on p. 98.

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FOREWORD

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GROUP III: Hereditary Thrombocytopathic Purpuras Case 3: Glanzmann's Thrombasthenia Cases 4-5 Pseudohemophilia with Platelet Problems Table LIII B

Case 3 (NP) was a severe bleeder (7) with P_{02} Fam Hist (6) The plat count (230 000) was normal but clot retr (12) was absent and the bleeding time (9) and T T (8) were very significantly P_{02} Plat accel (16) was not tested and plasma proaccelerin (29) was a low normal (62%) Plat tpin (15) and ser serotonin (17) were normal (100%) Test 19 was Neg but serum antithr (20) seemed to be increased Prothr consumption (25) was normal (95%)

Conclusion This rare case seems to be one of the very few reported (ref [383]) which conform to Glanzmann's [188] 'hereditary hemorrhagic thrombasthenia'

Cases 4, 5 (AC and SC mother and daughter) presented a familial (6) disorder which corresponded most nearly to the classical descriptions [484 485 486 260 262] of pseudohemophilia (ref [383 453])

Case 4 was a 52 year old white woman giving a ++ history of bleeding tendency (7) since 19 years of age The chief manifestations were ecchymoses and a moderately severe post operative hemorrhage Bleeding time (9) and clotting times (10 11) were normal as was clot retr (12) Her plat count (14) was normal (238 000) but the plat tpin (15) significantly reduced (50%) and plat accel (16) a low normal (50%) Serot (17) NT The T T (8) was significantly +++ Prothr consumption (25) was normal (>95%) as were AHF (22) PTC (23) and other tests Plasma proaccel (29) and proconv (28) NT Fibr (21) was elevated

Case 5 the daughter 20 years old had also suffered ++ ecchymotic bleedings Her plat count (14) was a low normal (104 000) and the plat tpin (15) 25% and plat accel (16) 20% unequivocally reduced Serot (17) NT Plasma proaccel (29) was normal (90%) AHF (22) likewise and probably PTC (23) 60% Prothr consumption (25) was quite normal as were other tests including a slightly questionable (71%) (recip see p 57) prothr time (26) Clot retr (12) was a satisfactory +++ Fibr (21) was elevated

Discussion In these two cases of pseudohemophilia the platelet problems were relatively minor and functional (thrombocytopathic) They must be regarded as of questionable clinical significance in a type of hereditary disorder which appears to be primarily a vascular dysfunction We had six other cases of pseudohemophilia two of hereditary telangiectasia and two with bleeding hemangiomata none of whom had any platelet problems and whose tests therefore are irrelevant to the present inquiry

Acquired deficiencies of platelet functions will be presented in the other groups The uremia cases (Group VIII) are especially interesting (p 136)

GROUP IV: Acquired Thrombocytopenia (Cases 6-30) Table LIV

These 25 cases were clinically diagnosed as follows:

statistically determined on significantly large groups usually more than 50 cases

(B) Mothers The 32 cases studied permitted venepuncture during parturition. They all appeared healthy and well and gave no history of recent infections, antibiotic or excessive salicylate therapy, dietary inadequacy or gastrointestinal disturbance. The obstetrical anesthesia was non-contributory. Pregnancy, labor and the puerperium were uncomplicated by toxemia, hemorrhage or thromboses.

(C) Infants The 32 newborn babies yielded blood samples from the umbilical cord before it had stopped pulsating. All babies were delivered without trouble, were uninjured and appeared normal. None developed any bleeding tendency. Some mothers and some babies received vitamin K, questions concerning which are answered in the cited references [172-173].

Test Results See Table LII. For purposes of this thesis comment will be limited to the noting of the absence of any platelet problems in (B) and (C). All tests in this regard fall within normal (A) limits. A single exception (10%) of low platelet accelerator (16) in (C) was of doubtful significance (possible lability).

The many interesting findings in other tests are fully dealt with in the cited publications and include data on another 125 newborns and most of their mothers, which are not included in the present more completely studied group.

GROUP II Congenital (Familial) Thrombocytopenia (Cases 1-2) Table LIII A

Because of their unusual interest (ref. [453]) these cases will be reported individually in Table LIII A.

Case 1 (A B) was examined on two occasions: (a) February 1, 1954, before treatment and (b) April 20, 1955, a few days after splenectomy. In tests (a) the platelet count (14) was very low (38,000) and assays for specific platelet factors (15-18) were not attempted. Clot retraction (12) was almost absent but prothrombin consumption (25) was amazingly normal. The child had bleeding symptoms (7) at this time, dating back to shortly after birth. Bleeding time (9) was very prolonged and the T T (four nique test) (8) was strongly Pos.

Family histories (F H) (6) will not be detailed in the present studies but were significantly Pos in Cases 1 and 2.

In tests (b) after splenectomy all test values were restored to normal with the questionable exception of a 50% platelet tptn (15). The patient developed no further bleeding problems in a 12-month followup.

Case 2 (D D) was not a bleeder (7) despite the Pos Fam Hist (6). Nevertheless his platelet count (14) was low (98,000) and tests 8, 12, 15, and 16 were significantly abnormal. He should therefore be regarded as a potential bleeder.

Discussion Case 31 (R G) was a 63 year old colored man with primary adenocarcinoma of the stomach and generalized metastases. At autopsy the bone marrow was found to be hyperplastic with a greatly increased number of megakaryocytes. He was examined four or five times between June 23 1953 and his demise on August 27 1954. The cited tests were on the first occasion but the thrombocytosis persisted at similar levels through June 29 1954. He developed a large hematoma in the upper right arm inexplicably. Except for a low platelet count (16) on January 6 1954 all tests of platelet function including clot retraction (12) and prothrombin consumption (25) were normal. Serotonin (17) was not tested however. The fibrinogen (21) was a borderline (200 mg) on January 6 1954 and plasma proaccel (29) low (20%) on the same occasion. These findings could go with a fibrinolytic enzyme activation which might have been overlooked in recording of a glass clot (10) of 1 min and ++++ clot retraction (12) on that occasion. We do not feel sure of the crude fibrinolysin test however unless all the clot dissolves within 24 hours.

Case 32 (J L H) was a 64 year old white male long known to suffer from polycythemia rubra vera. Cout was an interesting complication [ref [503]]. He had received P³² (red cell destructive) therapy at the Veterans' Hospital and we examined him twice (March 30 1954 and April 8 1955) after such courses of treatment when he was showing some bleeding symptoms [cf Group IX]. Despite the extremely high platelet counts (14) the platelet function tests (15 16) and especially (17) serotonin were significantly depressed. So were the prothrombin and related factors (26 29) but not the prothrombin consumption (25) although this might be misleadingly calculated because of the lowered level of the original plasma prothrombin.

Case 33: (E M) was a 23 year old white male leukemic whom we have excluded from Group VI solely because of the unusual thrombocytosis. Serotonin (17) was not tested but the other platelet function tests were normal. There was some hypoprothrombinemia (27) and prolonged prothrombin time (26) which might have to do with liver function. This case showed no hemorrhagic symptoms.

GROUP VI Leukemias with Thrombocytopenia or Thrombocytopathia Problems (Cases 34-67) Tables LVI A LVI B

Besides the thrombocytosis case (No. 33 Group V) platelet problems of one kind or another presented in 34 more (i.e. all but 4) out of 39 cases of leukemias studied. The remainder of these test data will be separated into two groups (A B) by arbitrarily dividing them at a 100 000/mm³ level of the platelet count.

Table LVI A presents a summary of the thrombocytopenics (22 cases).

Discussion Twelve of these cases recorded bleeding symptoms. (7) Platelet counts (14) ranged between 17 000 and 100 000. Platelet function tests (15 16 17) were significantly reduced in all but one or two. Platelet agglutination (18) was demonstrated in 5 out of 11 tested cases. The T T (8) was Pos in 5 cases and bleeding time (9) prolonged in 7 cases. Silicone clot (11) was significantly prolonged in 15 cases. There were 16 cases and one doubtful with defective prothrombin consumption (25). Antithrombin tests (19 20) were significantly altered in many especially if we include minor variations from the

- (A) 11 acute ITP (idiopathic thrombocytopenic purpura) of which 3 may have been due to drugs and 3 to infections namely (1) respiratory infection in a 17 mo old child (2) diphtheria in a 4½ yr old child and (3) infectious (? toxic) hepatitis in an 18 yr old white girl
- (B) 7 chronic ITP including one of two months duration associated with rheumatoid arthritis in a 15 year old girl
- (C) 5 were associated with anemias namely (1) two aplastic anemias (2) two macrocytic anemias one pernicious and the other occurring during pregnancy in a 22 year old woman and (3) one with Banti's syndrome
- (D) 2 were associated with metastatic (bone) carcinomata in elderly males with primary prostatic malignancy

Test results are given in Table LIV in summary

Discussion Plat counts (14) ranged from 5 000 to 84 000 except for the 174 000 (at this examination December 10 1953) in a 33 year old colored woman with pernicious anemia (y C) Many showed a severe bleeding tendency (7) one was doubtful 4 N T and only one gave no bleeding history The T T (8) was Pos in 19 and questionable in 2 others who were Negroes whose dark skin interfered with the reading of this test Bleeding time (9) was Pos in 17 and questionable in one Clotting time (glass 10) was definitely prolonged in only one case but the silicone c t (11) was Pos in 10 cases This indicates the superiority of test 11 in cases with borderline clotting time increases Clot retr (12) was Pos in 17 cases None showed fibrinolysis (13) Platelet functions (15 16 17) were usually reduced pari passu with the lowered platelet count (14) For (15) plat tpin testing 20 cases 19 were Pos and questionable in one For (16) plat accel testing 19 cases all 19 were Pos For (17) ser serotonin testing 12 cases 6 were Pos Prothr consumption (25) was defective in 16 and questionable in one This is highly significant but the quantitative correspondence between platelet levels and degree of failure to utilize prothrombin showed a very poor correlation It is suggested that the quality of the platelets in terms of functional capacities is more important than mere numbers in controlling prothrombin utilization The plasma components (22 23) of this mechanism were normal except for one borderline (60%) and one low normal (65%) PTC assays neither in the range which could be expected to influence the prothr consumption test

A small incidence (see Table) of other thrombin factor deficiencies is unexplained It is possible that liver function is occasionally disturbed perhaps by hemorrhage The elevated fibr (21) in 9 cases may be evidence of a liver reaction The low plasma proaccelerin (29) in 3 cases does not reflect the many more 19 cases low values for plat accel (16) Plasma thr c t (19) was definitely prolonged in 15 cases and slightly so in 8 more cases We do not yet know the possible significance of this finding

GROUP V Thrombocytosis (Cases 31 33) Table LV

These three cases present different clinical disorders but show in common very elevated platelet counts (14) Two 31 and 32 had bleeding symptoms

Results are detailed in Table LV

Discussion Case 31 (R G) was a 63 year old colored man with primary adenocarcinoma of the stomach and generalized metastases. At autopsy the bone marrow was found to be hyperplastic with a greatly increased number of megakaryocytes. He was examined four or five times between June 23 1953 and his demise on August 27 1954. The cited tests were on the first occasion but the thrombocytosis persisted at similar levels through June 29 1954. He developed a large hematoma in the upper right arm inexplicably. Except for a low platelet (16) on January 6 1954 all tests of platelet function including clot retraction (12) and prothrombin consumption (25) were normal. Serotonin (17) was not tested however. The fibrinogen (21) was a borderline (200 mg) on January 6 1954 and plasma proaccel (29) low (20%) on the same occasion. These findings could go with a fibrinolytic enzyme activation which might have been overlooked in recording of a glass clot (10) of 15 min and ++++ clot retraction (12) on that occasion. We do not feel sure of the crude fibrinolysin test however unless all the clot dissolves within 24 hours.

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marked in the less mature megakaryocytes. They were not observed in polykaryocytes (osteoblasts) which are unrelated to platelet origin.

(8 D) THROMBOCYTES [139]

The nucleated thrombocytes of amphibia (frog) reptiles (turtle) fish (fresh water sun fish) and birds (fowl) have been studied under similar conditions. Plate 7 (Figure 19) shows an altered frog thrombocyte lying on top of a large fibrin filament. Note the vesicular bodies derived from the thrombocyte some still attached and others released for some distance. As frequently noted in the earlier literature on thrombocytes their disintegration is often very sudden and explosive in character. This scatters excrescences chiefly vesicles and granular material to some distance from the parent thrombocyte as well shown in Plate 8 (Figure 19) in the case of the sun fish observation. It is noteworthy however that many vesicles persist in the region of the original adherence and disintegration of the thrombocyte. The dark field observations confirm the essential similarity of the alterations of thrombocytes megakaryocyte margins and mammalian platelets.

(8 E) ERYTHROCYTE STROMATOLYSIS ETC

Furchgott [182] in 1940 made dark field observations of altering erythrocyte 'ghosts' (stroma of hemolyzed red cells) in contact with lyotropic salt solutions such as lithium perchlorate or potassium thiocyanate. He was apparently unaware of our earlier data on platelet alterations and independently suggested the myelin figure explanation of the phenomena observed. His search of the red cell literature revealed several earlier observations (e.g. Oliver [355] Kite [267] Auer [23] and others) of essentially similar appearances from both hemolyzed and non hemolyzed red cells. In some observations made by the present author in Cape Town in 1930 and recorded in the photomicrograph reproduced in Plate 9 Figure 20 'stromatolytic filaments' were observed extending from the margins of young erythroblasts in a drop of heart blood from a rabbit embryo. Plate 11 Figure 20 photographed by an erstwhile graduate student (P. H. Ralph) working under the author's supervision at the University of Michigan in the early 1940's shows apparently similar thread like processes connecting points at which normal adult human red cells have apparently touched and then withdrawn from one another. Plate 10 Figure 20 also prepared by Dr. P. H. Ralph is a striking example of the spiculated stromatolytic appearances occurring in vitro in slide (dark field microscopy) observations of a nucleated red cell from a tadpole [139].

Conclusion. There is clearly a common phenomenon connected with surface alterations of the several varieties of blood and bone marrow formed elements collectively considered in the foregoing observations. The basis for attributing these phenomena to a myelin figure formation from the surface phospholipids of these formed elements follows from the next series of observations.

(8 F) MYELIN FIGURE FORMATIONS FROM PHOSPHOLIPIDS

R Virchow [483] first described myelin forms (figures as microscopical appearances at the edges of nervous tissue observed in contact with watery solutions). He interpreted them in terms of surface (interphase) relations between the lipid and aqueous phases. J. B. Leathes [284] studied them very extensively in 1925. He photographed at successive $\frac{1}{2}$ minute intervals the large myelin figures appearing at the edge of a film of lecithin in contact with a wide variety of watery solutions chosen to illustrate the effects of (a) specific ions (b) osmotic concentration and (c) adsorbed substances. The myelin forms were readily produced by distilled water. Leathes noted a suppressive or inhibitory effect of calcium ions said to be overcome by 0.01M NaOH and by cholesterol. Other retarding and many favoring agents were recorded by this observer. The present author in 1934 [124] reproduced some of the lecithin experiments of Leathes. Plates 12A and 12B (Figure 20) show the dark field appearance (400x magnif.) of the edge of the phospholipid film. (A) at the start in contact with air showing only the linear highly refractile margin and (B) 3 minutes after contact with distilled water showing the typical large (initial) myelin forms flowing out into the watery phase (upper right part of photo). Clearly visible in the original but poorly reproduced in the photomicrograph (and therefore retouched) is the translucent border as described by Leathes [284].

Our observations established one or two new points which we believe to be of considerable significance.

Firstly all watery solutions cause an initial myelin figure outflow (i.e. into the aqueous phase) which is followed by a further expansion after a 5-10 minute 'delay period'.

Secondly the dark field method permits interpretation of Leathes' translucent border as a reciprocal phenomenon namely a penetration of the water into the lipid phase. The secondary expansion phase we observed to be very dependent upon the particular ions in the watery solution. If distilled water or sodium chloride (and similar salt) solutions were used the 'secondary myelin figure outflow went on with increasing speed and vigor but the inflow of watery phase into the lipid was limited and showed little further penetration beyond that seen in the initial phase. With calcium salt solutions these phase relations were reversed. There was the same initial myelin figure outflow but little extension after the delay period. Secondly however the inflow of this solution into the lipid proceeded apace and without limit extending the 'translucent border' throughout the lipid portion. Our important conclusion was that calcium is specific in directing water into the lipid phase as is well known from the work of physical chemists with phase relations of calcium and sodium salts in soaps and other fat water emulsions.

To Leathes' conclusion that myelin forms are due to surface growth and localized imbibition of water we would add that calcium ions specifically generalize the imbibition and cause a reverse myelin figure appearance as can be seen very clearly in dark field observations of lecithin films.

Applying these ideas to platelet (etc) alterations we conclude that the calcium has the specific role not of causing the excrescence formations (which can occur in citrate etc) but in the liquefaction of the excrescences. By directing water into the altering platelets or thrombocytes calcium plays an important role in their peculiar disintegrative phenomena. What about the nature of the lipid itself? In our 1945 observations [139] we were able to include some dark field observations on other phosphatides namely the purified preparations of Dr J Folch (of the Rockefeller Institute New York) which are described on p 98

The chief additional information obtained was that cephalin of the several phospholipids studied yielded myelin forms which in size and appearance e.g. vesicles were often so similar to the above described platelet megakaryocyte and thrombocyte alterations that it seemed reasonable to regard them as identical

Actually the fundamental myelin figure phenomenon was common to most of these phosphatides in contact with watery solutions and calcium salts caused a granular deposition penetrating into the lipid and interfering with its hydrotropic dispersion into the watery phase. The strength of the salt solutions had only minor effects suggestive of some membrane phenomenon in which water penetration rates are partly controlled by osmotic considerations

The inositol phosphatide (III) behaved peculiarly in that it went directly into colloidal suspension as 'chylomicra' instead of showing 'myelin forms'. This is evidently representative of the behavior of the bulk of lipoidal and lipoprotein materials of plasma

Concerning the protein components of the surfaces of platelets etc our experiments have nothing to say

(8 G) CONCLUSION

In summary then our comparisons of the dark field microscopical appearances of (a) water phospholipid films and (b) in vitro alterations of platelets megakaryocytes thrombocytes and certain erythrocytes indicates the fundamental importance of myelin forms (figures) and related phenomena at water lipid interfaces. We believe these to be extremely primitive and essentially non vital phenomena of lipid rich surface membranes. Not only is there an hydro tropism of the lipid material causing the appearance of surface 'excrescences' of the various types described but there is also a specific role for the calcium ion. This reverses the phase relationships and directs water into the fatty phase thus contributing to the peculiar disintegrative phenomena studied. Cholesterol proteins osmotic factors pH and perhaps other factors need additional study as further possible modifiers of the basic phenomena which we have described

G CLINICAL CASES WITH PLATELET PROBLEMS

FOREWORD

Our research problem 1950-55 with regard to the investigation of clinical cases with hemorrhagic and related disorders was largely planned and executed by Dr Jessica H Lewis Research Associate

and Co investigator with advice assistance and some participation by the author. Invaluable technical assistance was rendered by Francis M Morgan Doris C Ferguson Iris Rudin Ann C Howe and Bertha G Jackson. Since 1953 a graduate student Mr James W Fresh has made many able contributions especially to Group I studies (see below) and in the past year Miss Mary Jane Patch has been a most helpful collaborator. The staffs of the University's (North Carolina) Memorial Hospital Duke Hospital the Veterans' Hospital and ORINS Hospital have cooperated with the patient material as have a number of private practitioners. We are indebted to Dr Marjorie B Zucker of the N Y U College of Dentistry Physiology Department for the serum serotonin assays [519] used to assess the platelet vasoconstrictor factor (Item 17 see Tables).

In preparing the data for this thesis the author has proceeded independently and has not consulted Dr Lewis who now has her own Research Laboratory at the University of Pittsburgh. Gratefully acknowledging the leading role of Dr Lewis and the individual contributions of each member of our group the author nevertheless assumes sole personal responsibility for the following presentation and analysis of the data selected.

CASES represent some 86 patients and 32 normal mothers and their infants. They are classified into nine groups. No attempt will be made to analyze them from the clinical viewpoint. Rather they represent merely a physiological contribution of analytical laboratory methods with which to approach a rational diagnosis and understanding of the clinical hemostatic problems. Our groups' are chosen from this physiological viewpoint to serve the purpose of this thesis inquiry.

Methods for the most part have been described on pp 53-62 and will not be further elaborated. An itemization (1-30) facilitates organization and reference. In the first Table (LII) the mean and range of test values are given for (A) normal healthy adults (randomized as to sex and age) (B) parturient mothers and (C) their normal newborn infants (cord bloods).

0 \pm (i.e. trace or minor) + ++++ indicate relative degrees of (7) bleeding tendency and (12) clot retraction (in glass). Retraction was also observed in silicone (cf 11) but this will not be reported since the differences from (12) were relatively minor. In group summaries the number of cases was divided for each test into the following categories:

Pos significant alteration of the test value

Neg not significantly different from the normal (norm.)

? doubtfully significant

N.T.: no test performed

Results are presented in Tables LII-LX.

GROUP I (A) Normal Adults (B) Parturient Mothers (C) Normal Newborn Infants (Cord Bloods) ref [172] Table LII

(A) Normals were from healthy adults randomized as to sex and age. They were mostly medical students staff and hospital personnel including a few colored helpers. The means of the various tests were

statistically determined on significantly large groups usually more than 50 cases

(B) Mothers The 32 cases studied permitted venepuncture during parturition. They all appeared healthy and well and gave no history of recent infections, antibiotic or excessive salicylate therapy, dietary inadequacy or gastrointestinal disturbance. The obstetrical anesthesia was non-contributory. Pregnancy, labor and the puerperium were uncomplicated by toxemia, hemorrhage or thromboses.

(C) Infants The 32 newborn babies yielded blood samples from the umbilical cord before it had stopped pulsating. All babies were delivered without trouble, were uninjured and appeared normal. None developed any bleeding tendency. Some mothers and some babies received vitamin K, questions concerning which are answered in the cited references [172-173].

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The many interesting findings in other tests are fully dealt with in the cited publications and include data on another 125 newborns and most of their mothers, which are not included in the present more completely studied group.

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Because of their unusual interest (ref. [453]) these cases will be reported individually in Table LIII A.

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Family histories (F H) (6) will not be detailed in the present studies, but were significantly Pos in Cases 1 and 2.

In tests (b) after splenectomy, all test values were restored to normal, with the questionable exception of a 50% platelet pln (15). The patient developed no further bleeding problems in a 12-month followup.

Case 2 (D-D) was not a bleeder (7) despite the Pos Fam Hist (6). Nevertheless, his platelet count (14) was low (98,000) and tests 8, 12, 15, and 16 were significantly abnormal. He should therefore be regarded as a potential bleeder.

GROUP III: Hereditary Thrombocytopathic Purpuras Case 3 Glanzmann's Thrombasthenia Cases 4 5 Pseudohemophilia with Platelet Problems Table LIII B

Case 3 (N P) was a severe bleeder (7) with Pos Fam Hist (6) The plat count (230 000) was normal but clot retr (12) was absent and the bleeding time (9) and T T (8) were very significantly Pos Plat accel (16) was not tested and plasma proaccelerin (29) was a low normal (62%) Plat tpin (15) and ser serotonin (17) were normal (100%) Test 19 was Neg but serum antithr (20) seemed to be increased Prothr consumption (25) was normal (95%)

Conclusion This rare case seems to be one of the very few reported (ref [383]) which conform to Glanzmann's [188] 'hereditary hemorrhagic thrombasthenia'

Cases 4, 5 (A C and S C mother and daughter) presented a familial (6) disorder which corresponded most nearly to the classical descriptions [484 485 486 260 262] of pseudohemophilia (ref [383 453])

Case 4 was a 52 year old white woman giving a +(+ history of bleeding tendency (7) since 19 years of age The chief manifestations were ecchymoses and a moderately severe post operative hemorrhage Bleeding time (9) and clotting times (10 11) were normal as was clot retr (12) Her plat count (14) was normal (238 000) but the plat tpin (15) significantly reduced (50%) and plat accel (16) a low normal (50%) Serot (17) N T The T T (8) was significantly +++ Prothr consumption (25) was normal (>95%) as were AHF (22) PTC (23) and other tests Plasma proaccel (29) and proconv (28) N T Fibr (21) was elevated

Case 5 the daughter 20 years old had also suffered +(+ ecchymotic bleedings Her plat count (14) was a low normal (104 000) and the plat tpin (15) 25% and plat accel (16) 20% unequivocally reduced Serot (17) N T Plasma proaccel (29) was normal (90%) AHF (22) likewise and probably PTC (23) 60% Prothr consumption (25) was quite normal as were other tests including a slightly questionable (71%) (recip see p 57) prothr time (26) Clot retr (12) was a satisfactory +++ Fibr (21) was elevated

Discussion In these two cases of pseudohemophilia the platelet problems were relatively minor and functional (thrombocytopathic) They must be regarded as of questionable clinical significance in a type of hereditary disorder which appears to be primarily a vascular dysfunction We had six other cases of pseudohemophilia two of hereditary telangiectasia and two with bleeding hemangiomata none of whom had any platelet problems and whose tests therefore are irrelevant to the present inquiry

Acquired deficiency of platelet functions will be presented in the other groups The uremia cases (Group VIII) are especially interesting (p 136)

GROUP IV: Acquired Thrombocytopenia (Cases 6 30) Table LIV

These 25 cases were clinically diagnosed as follows

- (A) 11 acute ITP (idiopathic thrombocytopenic purpura) of which 3 may have been due to drugs and 3 to infections namely (1) respiratory infection in a 17 mo old child (2) diphtheria in a 4½ yr old child and (3) infectious (? toxic) hepatitis in an 18 yr old white girl
- (B) 7 chronic ITP including one of two months duration associated with rheumatoid arthritis in a 15 year old girl
- (C) 5 were associated with anemias namely (1) two aplastic anemias (2) two macrocytic anemias one pernicious and the other occurring during pregnancy in a 22 year old woman and (3) one with Banti's syndrome
- (D) 2 were associated with metastatic (bone) carcinomata in elderly males with primary prostatic malignancy

Test results are given in Table LIV in summary

Discussion Plat counts (14) ranged from 5 000 to 84 000 except for the 174 000 (at this examination December 10 1953) in a 33 year old colored woman with pernicious anemia (y C). Many showed a severe bleeding tendency (7) one was doubtful 4 NT and only one gave no bleeding history. The T T (8) was Pos in 19 and questionable in 2 others who were Negroes whose dark skin interfered with the reading of this test. Bleeding time (9) was Pos in 17 and questionable in one. Clotting time (glass 10) was definitely prolonged in only one case but the silicone c t (11) was Pos in 10 cases. This indicates the superiority of test 11 in cases with borderline clotting time increases. Clot retr (12) was Pos in 17 cases. None showed fibrinolysis (13). Platelet functions (15 16 17) were usually reduced pari passu with the lowered platelet count (14). For (15) plat tpin testing 20 cases 19 were Pos and questionable in one. For (16) plat accel testing 19 cases all 19 were Pos. For (17) ser serotonin testing 12 cases 6 were Pos. Prothr consumption (25) was defective in 16 and questionable in one. This is highly significant but the quantitative correspondence between platelet levels and degree of failure to utilize prothrombin showed a very poor correlation. It is suggested that the quality of the platelets in terms of functional capacities is more important than mere numbers in controlling prothrombin utilization. The plasma components (22 23) of this mechanism were normal except for one borderline (60%) and one low normal (65%) PTC assays neither in the range which could be expected to influence the prothr consumption test.

A small incidence (see Table) of other thrombin factor deficiencies is unexplained. It is possible that liver function is occasionally disturbed perhaps by hemorrhage. The elevated fibr (21) in 9 cases may be evidence of a liver reaction. The low plasma proaccelerin (29) in 3 cases does not reflect the many more 19 cases -low values for plat accel (16). Plasma thr c t (19) was definitely prolonged in 15 cases and slightly so in 8 more cases. We do not yet know the possible significance of this finding.

GROUP V Thrombocytosis (Cases 31 33) Table LV

These three cases present different clinical disorders but show in common very elevated platelet counts (14). Two -31 and 32 had bleeding symptoms.

Results are detailed in Table LV

Discussion Case 31: (R G) was a 63 year old colored man with primary adenocarcinoma of the stomach and generalized metastases. At autopsy the bone marrow was found to be hyperplastic with a greatly increased number of megakaryocytes. He was examined four or five times between June 23 1953 and his demise on August 27 1954. The cited tests were on the first occasion but the thrombocytosis persisted at similar levels through June 29 1954. He developed a large hematoma in the upper right arm inexplicably. Except for a low platelet accel (16) on January 6 1954 all tests of platelet function including clot retri (12) and prothr consumption (25) were normal. Serotonin (17) was not tested however. The fibrinogen (21) was a borderline (200 mg) on January 6 1954 and plasma proaccel (29) low (20") on the same occasion. These findings could go with a fibrinolytic enzyme activation which might have been overlooked in recording of a glass c t (10) of 1¹/₂ min and ++++ clot retri (12) on that occasion. We do not feel sure of the crude fibrinolysin test however unless all the clot dissolves within 24 hours.

Case 32 (J L H) was a 64 year old white male long known to suffer from polycythemia rubra vera. Cout was an interesting complication (ref [503]). He had received P³² (red cell destructive) therapy at the Veterans' Hospital and we examined him twice (March 30 1954 and April 8 1955) after such courses of treatment when he was showing some bleeding symptoms (cf Group IX). Despite the extremely high platelet counts (14) the platelet function tests (15 16) and esp (17) serotonin were significantly depressed. So were the prothrombin and related factors (26 29) but not the prothr consumption (25) although this might be misleadingly calculated because of the lowered level of the original plasma prothrombin.

Case 33: (E M) was a 23 year old white male leukemic whom we have excluded from Group VI solely because of the unusual thrombocytosis. Serotonin (17) was not tested but the other platelet function tests were normal. There was some hypoprothrombinemia (27) and prolonged prothr time (26) which might have to do with liver function. This case showed no hemorrhagic symptoms.

GROUP VI Leukemias with Thrombocytopenia or Thrombocytopenia Problems (Cases 34 67) Tables LVI A LVI B

Besides the thrombocytosis case (No 33 Group V) platelet problems of one kind or another presented in 34 more (i.e. all but 4) out of 39 cases of leukemias studied. The remainder of these test data will be separated into two groups (A B) by arbitrarily dividing them at a 100 000/mm³ level of the platelet count.

Table LVI A presents a summary of the thrombocytopenics (22 cases).

Discussion Twelve of these cases recorded bleeding symptoms (7). Platelet counts (14) ranged between 17 000 and 100 000. Platelet function tests (15 16 17) were significantly reduced in all but one or two. Platelet agglutination (18) was demonstrated in 5 out of 11 tested cases. The T T (8) was Pos in 5 cases and bleeding time (9) prolonged in 7 cases. Silicone c t (11) was significantly prolonged in 15 cases. There were 16 cases and one doubtful with defective prothrombin consumption (25). Antithrombin tests (19 20) were significantly altered in many especially if we include minor variations from the

normal controls. Twelve and one questionably had a low plasma proaccelerin (29) but half again as many cases had low plat accel (16). Seven had prolonged prothr time (26) but the specific thrombin forming factors (except for the accel noted) were usually normal or nearly so.

Table LVI B summarizes leukemias with normal (or nearly normal) platelet counts but with evidence of some or other deficiency of platelet functions details of which can be noted in the table. Five of these 12 thrombocytopathia cases recorded bleeding problems (7).

In addition to the tests reflecting platelet functions (8, 9, 12, 14, 18, 25) a prolonged silicone c t (11) might be significant in some instances. As compared with the A group's 17/22 the B group's 5/12 is a smaller proportion showing defective prothr consumption (25).

Conclusion It would appear that platelet defects, whether definite thrombocytopenia or functional platelet defects (thrombocytopathias) are commonly encountered in leukemias. This may very well play a leading role in the hemorrhagic tendency in leukemia. Cases with abnormal values in our tests but no overt bleedings may be regarded as potentially liable to episodes of bleeding.

Some less frequent reductions in other clotting factors (e.g. prothrombin, etc.) may reflect injury to liver as well as bone marrow functions.

Addendum While both acute and chronic types of the common myeloid and lymphatic leukemias are included in both sub groups, the A series contained relatively more of the acute types while the B series had more chronics. Included in A also were (1) one case of coincident polycythemia which is now a well recognized [503] association and (2) one case which was finally diagnosed as lymphosarcoma. Included in B was one case of monocytic leukemia.

GROUP VII Platelet Problems in Uremia (Cases 68-78) Tables LVII A, LVII B

Out of 12 uremic cases studied because of bleeding problems, 11 showed various platelet anomalies. These are summarized in Table LVII.

(A) Cases 68-77 In this sub group of 10 cases, 8 had definite bleeding problems, 1 denied such, and 1 lacked the necessary history (7). The findings itemized 8-12 were Neg or infrequent. Platelet counts (14) were all within the normal range. Quite otherwise however were many of the platelet function tests. Thus plat tpin (15) was low in 7 cases and plat accel (16) in 7 and one doubtful. The two cases tested for serotonin (17) gave significantly low values. Four cases and one doubtful showed defective prothrombin consumption (25) with insufficient lowering of the tested plasma components to account for this.

Conclusion We believe these data offer a new explanation for the bleeding tendency in uremics, namely that their platelets, while normal in number, are functionally defective (thrombocytopathic) much as they are in many leukemias (Table LVI B). The not infrequent defect in prothrombin consumption could be a result of this also.

(P) Case 78 is of doubtful interest. The one borderline (50%) value for plat accel (16) on the first of the two examinations is the only

justification for its inclusion in Table LVIII. Platelet counts and other functional platelet tests were normal on the two occasions. The clinical diagnosis of chronic pyelonephritis in polycystic kidneys showed an infectious complication of an already toxemic nitrogen retention. The low levels of PTC and thrombin forming factors on May 30, 1955, the day before demise, could have been due to liver failure. We would just be guessing to blame her bleedings on some toxic disturbance to vascular integrity.

GROUP VIII Platelet Problems in Cases with Plasma Clotting Difficulties (Cases 79-85) Table LVIII

These 7 cases (including one questionable) represent a very small minority of our collected data on 52 hemophiliacs, 26 PTC deficient, 24 acquired hypoprothrombinemias, 5 congenital hypoproconvertinemias, 4 congenital hypoproaccelerinemias, 8 cases with circulating inhibitors (anti AHF or anti PTC) and several others with obscure clotting problems. The data of the 7 cases are individually analyzed in Table LVIII.

(A) Case 79-83 might be subgrouped as primarily severe liver disorders with mild systemic bleeding problems. Two (80-81) had marked jaundice and four (80-83) showed no response to their hypoprothrombinemia to vitamin K. Platelet counts (14) in Nos. 79-82 were considerably reduced (31,000-92,000) and it was questionably low (128,000) in Case 83. Functional tests were N.T. on Case 83 but were significantly low for (15) tptn and (16) platelet accel. in the four others. Serum serotonin (17) was quite normal in the same cases, however. The T.T. (8) was + in Case 80 but neg in 79-81-82. Clot retr. (12) was only ++ in Case 82 but normal in 79. Lysis (see below) interfered in the other cases. The prothrombin consumption (25) was quite normal in 79-82 and N.T. in 83. Plasma thromboplastin (19) was increased in the two specimens (79-82) in which there was no interfering fibrinolysis. Serum antithrombin (20) tested normal in 3 cases. The liver dysfunction could account for the low levels of PTC (23), prothrombin (26-27), proconvertin (28) but not necessarily for the proaccelerin (unless the liver has a 'storage' function for this component of the thrombin forming system [322-492]). Fibrinolysis was a complication which could also be explained by the severe liver injury [453]. It interfered with determinations of clotting times (10-11), clot retr. (12) and plasma thromboplastin (19). A borderline (200 mg) fibrin (21) in case 83 may have been due in part (?) to the fibrin(ogen)olysin but the fibrinogen was slightly above normal in Case 81 who also showed fibrinolysis.

(B) Case 84 (DH) was a 3 year old white female child who was the most severe of the 4 congenital hypoproaccelerinemia cases whom we have studied and was the only such case with unusual platelets. The absence of proaccelerin (29) sufficiently explains the bleeding tendency (~) the prolonged clotting (10-11) and prothrombin time (26) as well as the defect (43%) in prothrombin consumption (25). True prothrombin (27), proconvertin (28), PTC (23), AHF (22) and fibrin (21) were all normal. There was no fibrinolysis (13) nor inhibitor (24) and clot retr. (12) was normal. The platelet count (14) was a normal 240,000 and platelet tptn (15) normal (100%). However the platelet accel. (16) was very low indeed although it was measurable. The high value (250%) for serum serotonin is interesting and the suggestion that this might be a physiological

'compensation' is an open question. It was this case that most strongly influenced us toward a tentative hypothesis that the platelet accelerator is very closely related to if not identical with plasma proaccelerin.

(C) Case 85 (C R) was a 70 year old white male with prostatic carcinoma and metastases. His bleeding problem was primarily associated we believe with a circulating fibrinolysin (ref [453]). When first seen (January 10 1955) the plat count (14) was low (36 000) as were plat tpin (15) only 6 μ and plat accel (16) a barely assayable 1%. Plasma proaccel (29) was also very low and fibr (21) seriously depleted. Our experience with cases of active fibrinolysin supports the thesis that fibrinogen and accelerator factor are the two clotting factors most easily destroyed by the enzyme. At least in clinically encountered fibrinolysin concentrations the other clotting factors are resistant. If our interpretation is correct the enzyme in Case 85 is also attacking plat accel and perhaps whole platelets which could account for the anomalous platelet findings. The thrombocytopenia could explain the defective prothr consumption (25) and the lack of accelerator the prolonged prothr time (26). There was a significant increase in serum antifibrinolysin (24) which follows a pattern which we and others [453] have observed in such cases. The second examination seven weeks later showed the enzyme still to be present but in reduced amounts and with the same high antifibrinolysin titer (24). Platelets (14) had increased to practically normal and fibrinogen (21) and other tests (26-29) were considerably improved except for the important prothr consumption (25) which registered an unaccountable zero. AHF (22) was not tested at this time and it would have been interesting to have had this perhaps vital information.

GROUP IX Platelet Problems in Radiation Sickness (Case 86) Table LIX

(A) In humans our experience is confined to a single case R T (No 86) who was an elderly white male with a carcinoma of the colon which had metastasized to the liver. With such a hopeless prognosis he was being given radio gold (Au^{198}) at the ORINS Hospital (Oak Ridge Tennessee) and the author in consultation with the AEC was permitted to obtain the test data given in Table LIX.

Results refer only to two tests namely prothr consumption and plat counts [153] but they are very interesting. On the day (1) before the radio gold injection the patient was in poor shape and the prothr consumption at 67% (23% residual in 1 hr serum) was definitely defective although the plasma prothrombin was rather high at 510 two stage (p 59) units/ml tested with a very reactive fibrinogen ('normal' = 400-450 units). It could have been evidence of benefit to the liver that the prothrombin consumption returned to normal for two weeks after the Au^{198} injection. The platelet counts were essentially normal throughout this period as they were at the start.

The 19 day tests however showed the prothrombin consumption decreasing again (20% resid - 80% cons) while the platelets (158,000) were beginning to fall although the level at this time (on a purely numerical basis) should not have been able to account for the Poa prothr consumption test. The 22 day tests showed serious deficiencies of both platelets (48 000) and prothr consumption (55% resid = 45% cons).

On the following day the patient died from a massive hemorrhage at the primary carcinoma site. This could of course have been a vascular erosion but the defective hemostatic mechanisms were certainly a fatal handicap.

(B) Dog experiments

Experiment 50 Table LX

Purpose To inject dogs with Au^{198} intravenously in an effort to localize the bulk of the radiation ('hard γ and some β emissions) in the liver (which is known to sequester colloidal gold as used for these experiments). Effects on the coagulation mechanisms were to be investigated following such leads as appeared to be profitable and practicable under the rather restricted laboratory facilities made available to us due to the exactions of protective measures against the very 'hot dogs' who had to be isolated in outlying buildings devoid of the central laboratory's conveniences.

Methods The several test systems used were described in a publication [148] of these preliminary data on (2) two controls who received Au^{198} colloidal suspension with an old preparation which had lost all but a trace of its radioactivity and (b) five experimental animals who received 20 10 10 5 and a token 1.6 millicuries/kg Au^{198} fresh from the atomic pile respectively.

Results Table LX summarizes the day by day results for the prothr consumption and plat count tests on three of the animals (I II III).

Discussion Lethalities were as follows:

- (I); 20 mc/kg dose; death of animal on 9th day
- (II); 10 mc/kg dose; death of animal on 37th day
- (III); 5 mc/kg dose; animal surviving and apparently recovered 3 months later. Not illustrated are:
 - (IV) 10 mc/kg dose; apparent recovery after 4 weeks (according to pro cons test) but unexpected death on 83rd day
 - (V) 1.6 mc/kg dose; minimal effects and remained well until sacrifice on 106th day

The prothr consumption data and platelet counts given in Table LX showed some lack of correlation between the two tests. Thus the prothr consumption defect appeared in dogs II and III on the 6th day at which time the plat counts were still normal. Furthermore the prothr consumption test data were very different quantitatively on the 8th day in these same two animals although both platelet counts were moderately reduced and to about the same level.

Autopsy Findings

Dog (I); Liver was markedly icteric hemorrhagic and necrotic. Duodenum showed a section which nestled under the liver to be swollen with a massive interstitial hemorrhage. Hemorrhages were wide spread and particularly noteworthy were (1) bleeding into neck from the venepuncture prick of the previous day; (2) pulmonary hemorrhages (3) petchiae in serous membranes and in mucosa of alimentary canal (5) lymph node hemorrhages.

Dog (II): Liver showed acute damage reported as 'massive and complete hemorrhagic necrosis'. Hemorrhages were widespread including pulmonary

Dog (IV): Liver showed marked chronic damage reported as chronic cirrhosis with atrophy of liver cords engorged sinusoids and considerable hemosiderin apparently from old hemorrhage

Dog (V): Liver and other organs were normal
We are indebted to Dr G. A. Andrews for the autopsy reports and to Dr Marshall Brucer Head of the ORINS Medical Division for making these studies possible

Discussion The pathological data were supplemented with measurements of the residual radioactivity which confirmed the major concentration of the Au^{198} in the liver. There were however significant amounts also in the spleen lymph glands and bone marrow. Hence our preliminary attempt to localize internal irradiation for a particular organ (e.g. liver) by intravenous injection of a suitably chosen preparation was not altogether successful. What was remarkable however was the extent of liver damage which radiations thus achieved could cause without significant effects on prothrombin fibrinogen and other clotting factors.

No circulating (e.g. heparin like) anticoagulant could be detected in our experiments

The one test which these preliminary experiments supported as showing very significant results was the prothrombin consumption test (whether by 2 stage or 1 stage methods). What is of relevance to the present thesis is the apparent lack at times of correlation with the well known thrombocytopenic effect of radiations [14]. Jackson Cronkite et al [96 97 245 246] Penick et al [374] and others [477] have reported on the post irradiation thrombocytopenias and lack of effects on blood-clotting factors including AHF [374] although causing defects in prothrombin consumption. We accord great respect to these other workers particularly to Dr E. P. Cronkite for his work performed while a Commander in the USN including participation in animal experiments both at the Naval Research Laboratories (X irradiation) and in the test atomic bombings at Bikini and Eniwetok. Cronkite also noted discrepancies of the two tests especially during the recovery phase post irradiation.

We believe the evidence for the significant role of platelets as particularly established by Comdr Cronkite's group of workers to be most convincing. However we wish to keep an open mind on the possibility for some role also of certain plasma factors. We have plans for studying the PTC and 'Factor X' levels which we had hoped to carry out this year but the writing of this thesis has necessitated a delay which we hope to overcome in the near future.

Conclusion Thrombocytopenia is a very significant correlate of the hemorrhagic problem of radiation sickness. However the prothrombin consumption test appears to be a more sensitive index of the associated blood defect(s) and would seem to reflect with some accuracy the severity and probable outcome of the radiation injury [148]. The possibility of discovering radiation damage to some plasma factor or factors which work with the platelets in prothrombin utilization is still an open question. Cats rendered thrombocytopenic by X irradiation have been used to estimate platelet life span in crossed circulation experiments [283].

PART III

INTEGRATION OF IDEAS

A FORMULATION

An integration of ideas based on the data of this thesis permits detailed formulation of the following 'working hypotheses'

1 Thromboplastic lipid There is abundant evidence for a significant and essential role in the physiological clotting process of a factor or factors of a lipoidal nature probably cephalin. Its chief mode of action may be termed thromboplastic. This signifies that it participates along with Ca^{++} ions in the conversion of prothrombin to thrombin in the presence of Stuart factor (R. S. p. 27) proaccelerin and other co factors. It is probable that (a) the initiation of blood clotting is dependent upon the availability of the proper form of the postulated lipoidal thromboplastin and (b) it may need some special preliminary reaction of thromboplastin generation to present it in a suitable form to the thrombin forming reaction. It is questionable that tissue or plasma thromboplastins are true lipoproteins [87]. Further (c) there are in all likelihood certain naturally protective inhibitory mechanisms to be overcome before thrombin formation can proceed. On these we may comment as follows.

2 Natural inhibitors Heparin with its co factor is a possible inhibitor which can be demonstrated in experimental systems. However there is considerable question as to the artificiality of such test systems and the best modern evidence (p. 30) doubts that there is enough heparin in the normal circulating blood to prevent clotting. Nevertheless such a mechanism could prevail locally where Mast cells are abundant in and around blood vessel walls (Jorpes [258]). Also clinical [37, 448] and experimental [498, 255] conditions of true heparinemia have been shown to exist. Another possibility which has been particularly advanced by Tocantins et al. [475] is that certain lipoidal antithromboplastins exist in the blood and are increased in certain states e.g. hemophilia [472] and post irradiation [471]. Tocantins is a very careful worker but there have been criticisms of his techniques (e.g. the question of variable ionic strengths [196]) and the consensus of most American coagulationists is to minimize (though not to dismiss) the antithromboplastin idea particularly in hemophilia where the evidence for deficiency of a specific AHF or antihemophilic globulin (p. 25) is convincing.

3 Cephalin A number of workers have endeavored to characterize the thromboplastic lipid and its complexes and also the lipid anti thromboplastin(s). The present author has contributed in this area and current investigations are continuing with collaboration of biochemical colleagues. The best evidence to date merely confirms the original

discovery of Howell (1912) and Zak (1912) The data reproduced in this thesis are particularly strong support for this The assay method developed in the author's laboratory is extremely sensitive and can detect the activity of thromboplastic (cephalin like) materials at dilutions that run into the millions This means that such activity may be picked up in purified materials in which the real factor is merely a trace contaminant We believe that this may explain the doubts [84 88 76] that the thromboplastic lipid is really cephalin The chief basis of such doubts is the finding of activity in alcohol or acetone soluble lipid fractions That some cephalin may find its way into such fractions as a contaminant is a very reasonable supposition and it may very well be true that lipid solubilities are not an exact method of fractionation when dealing with the crude mixtures extracted with alcohol and ether benzene chloroform and other lipoidal solvents Hence we shall adopt as the current working hypothesis the conclusion that the essential thromboplastic lipids are true cephalins or ethanolamine phosphatides There is some old evidence (e.g. Gratia and Levene [200]) that the unsaturated fatty acids are necessary Lysocephalins are inactive [221 53 300 79] Certain synthetic cephalins containing only saturated fatty acids were found to be inactive (thromboplastically) by ourselves pp 98 99 confirming earlier work [202 263] A more recent synthetic cephalin prepared by Baer et al [28 29] and tested by our colleagues Dr K M Brinkhous and other members of his Pathology Department (University of North Carolina) [282] was found to be very slightly active Our tests (p 99) show this to be negligible suggesting a trace impurity Other biochemical problems noted on p 99 such as possible differences between α and β cephalins etc await further investigations

4 Lipid antithromboplastin Relatively crude cephalins extracted from brain and other tissues (see pp 25 71) are very satisfactory for blood clotting experiments [489] They do contain some antithromboplastic lipid however which limits their use at higher concentrations (p 88) and this may be the explanation (at least in part) of the experimental finding of a cephalin optimum

One interesting new possibility is opened up by the author's most recent data (p 99) namely that antithromboplastin may not be so much a single factor as a number of lipid fractions acting together perhaps synergistically

5 Complete'ness of thromboplastin. A new way to demonstrate 'thromboplastin generation. There is no doubt that aqueous or saline tissue extracts (thrombokinas or tissue thromboplastin) in most clotting systems have a definite superiority over any currently available phospholipid preparation The author's Experiment 29 p 100 comparing Dr Quick's rabbit brain thromboplastin with the analyzed equivalent of its extracted P lipids is a particularly striking example of this Dr Seegers in personal communications reported that his highly purified prothrombins were very poorly activated by cephalin as compared with tissue thromboplastin This the present author [156] could confirm (p 90) However there was some doubt whether Seegers' thrombin forming systems were adequately provided with AcG and other necessary cofactors It may be that the excellence of cephalin in our

latest eluate (prothrombin + proconvertin) systems with added AcG was due to 'thromboplastin generation' in conjunction with the PTC and significant though small amount of AHF present (p 107 and Table XXXVII). In our best experiments (p 77) there were no differences in thrombin yield but only a little difference in the rates of thrombin formation. Even this difference however disappeared (p 78) when more AHG (antihemophilic globulin) was added to the test system. These experiments represent a new way of demonstrating the phenomenon of 'thromboplastin generation'.

6 Intermediates We are not at all sure whether a specific complex (? lipoprotein) type of complete thromboplastin is a necessary preliminary to thrombin formation. With so many factors known to participate it would be difficult to avoid the surmise that a number of intermediates (see p 48) occur during the thrombin forming reaction. Exact identification of these and the sequence of their appearance offers a ripe field for future harvesting. Our own current conclusions favor one type of intermediary for which we believe the current experiments provide trustworthy evidence. We term this the Ca cephalin protein intermediary complex. The cited data indeed show (1) thrombin formation may be interrupted during its early phases by addition of decalcifying agents (oxalate citrate sequestrene or better cationic exchange resins) resulting not merely in arrest of the prothrombin activation but in a progressive inactivation to what appears to be an inert or non thrombin by product (? autoprothrombin). However when the thrombin is fully formed complete decalcification results in no significant effect on its coagulant potency. Unlike the postulated intermediary therefore true thrombin is not a calcium compound [265]. Of course the decalcification may prevent prothrombin activation when performed early enough so that no Ca ions are available for the activating processes.

(2) A new datum easily demonstrated (p 85) when using cephalin as the thromboplastic additive is that a similar progressive inactivation during the intermediate phase can be effected by using the very mild lipoidal extractive benzene. Again there is no such effect when the thrombin formation has been allowed to go on to completion. This we regard as evidence for participation in the intermediary of a free available mobile and loosely binding phospholipid (cephalin). We recall [149] the very poor activation by calcium alone of Howell prothrombin which our recent analyses (p 91) show not to be lacking in AcG proconvertin PTC or AHG. Such prothrombin preparations by actual lipid analyses contained significant amounts of cephalin. If such amounts were added to the system in the free form i.e. as purified cephalin suspension they would give excellent thrombin formation.

7 The cephalin availability theory This then is the basis of the author's 'cephalin availability theory' which postulates that the phospholipid thromboplastic factor must be liberated or made available in some way from its otherwise inert lipoprotein combinations. Protein bound phosphatides are ubiquitous in both tissues and plasma even hemophilic plasma [132]. How they become available to function as an essential thromboplastic factor in blood coagulation poses some very intriguing questions.

8 Thromboplastic enzymes Since 1939 the author and his colleagues have performed a very large number of experiments designed to explore the possibility that certain proteolytic enzymes may have some role in this availability of cephalin. In such action we term them thromboplastic enzymes. Experimentally the pancreatic protease trypsin (but not chymotrypsin) provides a considerable body of evidence as follows:

1) Trypsin addition facilitates the clotting of whole blood [107] decalcified plasmas (but not if there is too much oxalate or citrate [149]) even hemophilic blood [480] or hemophilic plasma fractions [132]

2) Trypsin acts by promoting the conversion of prothrombin to thrombin [112] but again provided that there is not too great excess of oxalate or citrate [149]

3) In the thrombin forming system trypsin acts best if there is sufficient calcium and some (but sub optimal) amount of cephalin or tissue thromboplastin. Our [156] most critical Experiment 32 p 105 indicates no effect of trypsin with adequate calcium unless a weak thromboplastin is also added.

Hence we conclude that trypsin is not thromboplastic in its own right (controverting Eagle & Harris [112]) but merely makes the phospholipid (cephalin) and sometimes even the calcium more 'available' for the thrombin forming reactions. A possible basis for this action and to explain minor activity in the absence of sufficient Ca and P-lipid is a postulated ability of trypsin to 'disaggregate' Ca and lipid from their normally inert protein combinations.

4) The antiprotease experiments (pp 113-116) clearly show loss of the thromboplastic action of trypsin parallel with removal of proteolytic activity by suitable treatment with these anti trypsin preparations. Controls (pp 117-119) show most of these antiproteases to be devoid of their own inhibitory effects on thrombin formation.

6) The trypsin clotting of completely platelet free plasma (p 67 Table V) cannot involve any platelet potentiation. This therefore calls for additional consideration of plasma sources of thromboplastic lipid which will be considered on p 145.

9 The fibrinolysin question Cited evidence from the author's experience has been given to show essentially similar experimental phenomena for the natural plasma protease fibrinolysin (plasmin or tryptase [143]). However there was a most significant exception (p 114) namely that these thromboplastic effects in platelet potentiation experiments (which system showed them most clearly) were not abolished by antiproteases. This must throw the gravest doubts [301] upon all the evidence concerning fibrinolysin and require a future investigation of some other unknown component of all our fibrinolysin preparations which could explain the findings independently of the protease proper. Until this is definitively settled we withhold any commitment as to a genuine clotting role for fibrinolysin which was prematurely postulated by the author [136] in 1943.

10 Platelets The occasional success in obtaining a completely platelet free plasma which will not clot on simple recalcification even in glass (p 66 Table IV) is the best evidence that platelets are not

mally essential for blood coagulation. Three observations however still leave room for doubt. Thus:

1) Why does this experiment so often fail even when the technical skill does not seem to be less than in the few experiments which succeed [90 472]? Is it just a matter of a little fragmented platelet material eluding the experimental effort or could it be that the plasma itself sometimes contains the trace amounts of thromboplastic factor needed? Tissue thromboplastin contaminants can certainly be ruled out in the careful performance of these experiments.

2) Why is it that not only platelets and tissue thromboplastin additives work but also and to a very significant extent cephalin (Table IV) and the proteolytic enzymes trypsin and fibrinolysin (Table V)?

A tentative answer could be that the common factor in all cases is the presence of available thromboplastic P lipid. Tissue thromboplastin must be adventitious but undoubtedly occurs in many injuries to blood vessels. It appears to be quite the most effective. The next best in our experiments is not platelets (much concentrated as these were) but purified cephalin. Could not (experimental) trypsin disaggregate some mobile cephalin from the plasma lipoproteins as suggested on pp. 116 144? Let us defer any conclusions about fibrinolysin because of the doubts (see above) as to its purity in the preparations employed to date. Pancreatic trypsin does not occur in the blood and we [293] believe the claims cited for such e.g. in pancreatitis are due to trypsin acting as a kinase activator of profibrinolysin.

3) Finally just what is the effect of a wettable surface? It is true (p. 38) that wettable surfaces and injured endothelium (p. 36) have significance in the platelet alterations which ordinarily precede blood clotting. However the effects noted in the platelet free system of Experiment 4 (p. 66) must be on some plasma component(s) (? Hageman). Might there not be a considerable labilization of plasma lipoproteins with release of some cephalin (thromboplastic P lipid) on surface contact? Could this not at times be the cause of the experimental failure noted in 2) above and such be an alternative explanation to that based upon postulated platelet fragments?

11 The trigger mechanism Correlating these speculations with those of Erkelens [118] p. 51 may it not indeed be possible naturally to initiate the clotting process with thromboplastic lipid (probably cephalin) from the plasma or plasma lipoproteins? This is a very intriguing question and upon its final answer will depend the elucidation of: (a) the still mysterious trigger mechanism which physiologically initiates blood clotting when needed for defensive hemostasis and which is obviously not in operation (at least overtly) in the circulatory blood and also (b) the true importance of the blood platelets.

12 Clinical disorders of platelets The platelets show evidence of their important hemostatic functions in clinical bleeding disorders. Only part of these functions concerns the clotting mechanism. Other hemostatic roles of the blood platelets are related to (1) their ability to aggregate and adhere to injured vascular endothelium to form cellular thrombi and (2) their participation in the provision of a vasoconstrictor factor which modern evidence (p. 39) suggests is serotonin or 5 hydroxytryptamine creatinine sulfate. Just how

important these two mechanisms may be is best illustrated by congenital afibrinogenemia and some other serious clotting deficiencies. The rare congenital afibrinogenemic [296] lacks the essential ingredient (fibrinogen) for clot formation. That such cases survive despite an overt hemorrhagic tendency must point to the great physiological effectiveness of platelet (cellular) and vascular hemostatic mechanisms. Worthy of passing mention also is the evidence of Comparative Zoology that the lower and phylogenetically older animal species developed a thrombocyte function long before a plasma clotting system evolved [189].

13 Myelin forms In the platelet alterations (p 38) after blood is shed and which presumably also occur intravascularly after vessel injury and in thrombotic conditions the author's dark field observations (pp 123-127) provide very suggestive evidence for a significant role of the platelet lipids. In particular it is concluded that the peculiar platelet excrescences are simply a variety of the long-known myelin figure formations. Of purified phospholipids examined with a similar microscopic technique cephalin presented appearances which often simulated the platelet alterations in very minute detail. These alternative phenomena are not peculiar to platelets but could be shown (pp 127-129) also in examinations of megakaryocytes, thrombocytes (of lower animals) and occasional erythrocytes undergoing stomatolysis. Furchgott [182] in 1940 also suggested the myelin figure interpretation for the last but apparently was unfamiliar with the author's 1934 platelet observations.

14 Preservation of platelet thromboplastic component Of the several factors which platelets may contribute to the blood clotting mechanism it would seem reasonable to conclude that the chief is its thromboplastic component. This is at least in part extremely stable as shown by Experiment 35 (p 108). J. L. Tullis [479] who is directing the platelet work of the Harvard Plasma Fractionation Commission notes (*op. cit.* p 148). During storage platelets undergo a gradual aging process. A loss of fibrils (*cf.* [124] excrescences) is one of the first morphological changes to be seen. Moreover there is a concurrent loss of clot retracting ability without loss of thromboplastic activity (Tullis' italics). Using as test system the ability of preserved platelets to restore coagulability to platelet poor plasma the Harvard scientists claim remarkable preservation of platelets on storage. The present author believes this needs to be reconsidered in view of evidence that many other platelet functions may be physiologically needed and most of them are highly labile. Platelets fractionated from human donors for clinical use should be used almost immediately and even then there is evidence that their therapeutic benefits are short-lived.

15 Potentiation of platelet thromboplastin The second major point about the thromboplastic action of platelets is its potentiation by antihemophilic factor, PTC, etc. (see p 108). Current thinking concerns a postulated 'thromboplastin generation' from some reaction (probably a series of reactions) between the platelets and the several plasma components. The evidence of workers with the thromboplastin

generation test points to need not only for calcium but also for factor V (proaccelerin) etc. If not for (pro)thrombin itself in thromboplastic generation. The types of experiment pursued in the present thesis have not explored the possibilities of thromboplastin generation as a separate phase i.e. prior to the activation of prothrombin to thrombin. However, Experiment 40 (p. 115) does show the important potentiation of platelet thromboplastic action by purified AHG, and Experiment 11 (p. 77) shows that cephalin can substitute for platelets in this phenomenon, whereas the complete thromboplastin (tissue tpn) in sufficient amounts is not improved by the plasma component addition.

Again we conclude that the phospholipid cephalin most probably is the stable factor in platelets. This is a weak and incomplete thromboplastin in its own right, apparently, but yields a complete thromboplastin in the presence of sufficient AHG, PTC, etc. Our eluates (Table VI) contain these plasma components, especially PTC, but could probably be freed, if necessary, from the contaminant trace of AHF. This present AHF contaminant explains why our eluates and the older Howell prothrombin were so well activated by cephalin, as compared say, with Seegers' more purified prothrombins.

Another potentiation of platelet thromboplastic action by a trace of thrombin (Experiments 36-37) is not easy to explain in our systems which are supplemented with AcG (accelerin) of the serum type, i.e. not the precursor proaccelerin. Thrombin is known [492-290] to play a role in the activation of proaccelerin to accelerin. This removes a lag phase in our two stage thrombin formation tests. Contrary to the claim of Seegers et al. that platelet accelerator is like serum AcG, Dr. Lewis and the author have noted many times that it shows the lag phase in two stage clotting systems, which we regard as evidence for proaccelerin (Seegers' plasma type AcG). Were it not for our added accelerin, therefore, thrombin could be effecting a change from the precursor form (proaccelerin) to the complete accelerator (accelerin) in the platelet component. It is still just possible that this is indeed the case and could account for the thrombin potentiation. Consistent with this possibility is the fact, which we [154] have several times demonstrated with our eluates, namely, increasing the AcG to an excess several times the equivalent of its strength in normal adult human plasma enhances thrombin formation with no clear cut evidence of any AcG optimum. Pending more data on the exact quantities of AcG (and pro AcG) in our systems, therefore, we conclude that the thrombin effect might be of the nature described. It is however conceivable that the mild proteolytic actions of thrombin could disaggregate phospholipid from the platelet lipoproteins, as we postulate in the case of the much more powerful proteolytic enzyme trypsin. Lacking concrete evidence at this time, such a concept is merely a tentative suggestion which could guide future enquiry.

B. FINAL SUMMARIZATION

The material in this thesis supports the idea that a major role in the natural clotting mechanism must be assigned to a group of factors which collectively form the 'thromboplastic' activator(s) which, in conjunction with ionized calcium, convert(s) the prothrombin (precursor) into the active thrombin needed for catalyzing the conversion of

fibrinogen to fibrin clot We have advanced evidence for

- 1) the importance of certain lipids (? cephalin);
- 2) the normal need in plasma clotting for platelets;
- 3) the particular significance of a platelet component which has many analogies to cephalin in the thromboplastic system;
- 4) potentiation of the thromboplastic actions of cephalin of platelets and of tissue thromboplastin (to some extent) by various experimental additives Part of this may be explained as a 'thromboplastin generation' through co participation of certain plasmatic components (anti hemophilic globulin PTC etc) Part however may be the result of certain proteolytic enzymes particularly trypsin 'disaggregating' bound forms of phospholipid from the normally unavailable lipoprotein combinations and thus rendering it 'available' for participation in the generation of thromboplastin and hence the conversion of prothrombin to thrombin;
- 5) possible Ca containing and lipid containing 'intermediates' in the thrombin forming reaction(s);
- 6) myelin figure formation as an explanation of 'alterations' of platelets and certain other formed elements such as thrombocytes megakaryocytes stromatolytic erythrocytes
- 7) the multiplicity of factors which platelets may contribute to the blood clotting and hemostatic mechanism;
- 8) the occurrence of many clinical bleeding disorders due to deficiency of platelet functions Thrombocytopenias denote deficiency of platelet numbers and hence of the total bulk of the platelet factors available in the body Thrombocytopathias are deficiencies of specific platelet components and can be clinically significant even when the platelet count is normal Bleeding in leukemias uremias etc may often be accounted for in these terms
- 9) the nature and modes of action of heparin and other 'antithromboplastic' inhibitors and of some anti proteases in relation to the mechanisms discussed;
- 10) the 'cephalin availability theory' of the author as a useful hypothesis to explain the importance of the natural thromboplastic phospholipid Lipid release from platelet tissue or possibly plasma (Experiment 4) sources may very well be the long obscure trigger mechanism' which initiates blood coagulation

PART IV

SUPPLEMENT

THE VERSATILE TWO-STAGE METHOD FOR QUANTITATIVE STUDY OF BLOOD CLOTTING FACTORS

In the period since completion of the dissertation in the Spring of 1956 to the writing of this supplement in the Fall of 1959 the author and his collaborators have published additional papers which are listed in the continuation bibliography appended. This supplement includes a selection of those portions of the recent research which are relevant to the topic of the dissertation. These will deal particularly with a significant new extension of the two-stage method. Originally developed as Method III¹⁰ (eluate technique) for assay of prothrombin as detailed in the main text this method has now been subjected to a series of refinements and modifications whereby it can be adapted to the quantitative study of any clotting factor. Such a method is somewhat empirical as regards standardization and choice of reagents of reference so that the percentage units are relative rather than absolute. However the technique is reasonably simple accurate and sensitive the results being remarkably reproducible. Its versatility is appreciated when by the appropriate modifications it can be used to study (a) each individual factor in the clotting system (b) inhibitors (c) effects of any additives and (d) possible intermediates in the thromboplastin and thrombin-forming reactions. Moreover it confirms and explains many other types of test systems and thus leads to a basic understanding of the blood coagulation mechanisms.

TECHNIQUE FOR THE VERSATILE TWO STAGE

All experiments are performed at a relatively unvarying room temperature of $26 \pm 3^{\circ}\text{C}$ in mixtures at pH 7.3 buffered with imidazole NaCl. Activation of prothrombin to thrombin occurs in a 5 ml mixture (incubate) of prothrombin-substrate (e.g. eluate) and additives all significant clotting factors in the mixture being under quantitative control.

At $\frac{1}{2}$ -1 minute intervals 0.2 ml samples of incubate are removed and added to 0.2 ml of a standard fibrinogen and the clotting time (C.T.) noted.

COMPLETE systems are optimal for all clotting factors whereas DEFICIENT systems lack just one factor all others being optimal or at least adequate and unvarying. To the latter system the missing factor can be added quantitatively thus securing data on its effects in relation to the progressive thrombin formation.

Substrate Prothrombin is usually supplied in the form of a plasma eluate. When oxalated plasma is available a BaSO_4 -citrate eluate is prepared by the method described in the main body of the dissertation. We prefer this but when only citrated plasma is available we are able to secure a satisfactory substrate by $\text{Al}(\text{OH})_3$ adsorption phosphate buffer elution and subsequent dialysis.

0.1 ml of these eluates (human, canine or bovine) in the routine 5 ml incubate mixture usually provides a suitable amount of prothrombin together with other eluted factors particularly Factor VII (proconvertin), Factor X (Stuart), Factor IX (PTC) and probably PTA etc. Significant trace contaminants are factor V (AcG), factor VIII (AHF), Hageman factor 7 etc. but optima (or adequacy) of these must be assured by suitable additives when necessary.

Additives Optimal calcium is regularly provided by adding 0.5 ml 0.15M CaCl_2 per 5 ml incubate. The tissue thromboplastin which we now use instead of Soluplastin (see thesis) is obtained from acetone dried human (autopsy) brain. 200-500 mg of the acetone-free dried brain powder is weighed and extracted with 10 ml saline (0.85% NaCl) for 15 min at 51-53°C (water-bath) with gentle agitation. The tube is then centrifuged for 5 min at 1500 r.p.m. and the supernatant suspension strained through gauze. Routinely 20 mg of phenol is added as a preservative and the suspension is usually stable in potency for about 8 weeks in the refrigerator at 4°C. The exact amount of brain powder is determined by testing several strengths of preparation in the Quick prothrombin time test on fresh normal human plasma. That preparation is selected which gives an 11 sec end-point in the Quick test. An optimal amount for the two-stage tests is determined and used routinely. The cephalin (standard prothromboplastin) which is substituted for the tissue thromboplastin in the system modified for study of the phospholipid component and its cofactors is now routinely prepared from the same human brain powder by the Bell-Alton [38] technique. The powder should give a negative Liebermann-Burchardt reaction for cholesterol. One gram of brain powder is extracted with successive 5-10 ml portions of reagent chloroform until a total of 50 ml of CHCl_3 has been used. The solution is filtered through a Buchner funnel, the residue dried by air jet and then finely homogenized in 50 ml saline. This concentrated 2 per cent stock solution may be frozen stored at 20°C for as long as 12 months without loss of potency. Imidazole buffered saline dilutions of the stock solution are freshly prepared or used within a few days (storage at 4°C in refrigerator). The optimal strength is carefully determined for a normal complete incubate system and rechecked from time to time in the course of a series of experiments. AcG The Ware-Seegers [492] method of preparing BaCO_3 -adsorbed beef serum for use as a source of factor V (AcG) is described in the thesis. This reagent frozen-stored in 1-2 ml lots at -20°C has been found to retain its potency for several years. A series of dilutions is tested to determine the optimum for each series of experiments. We have recently found that this AcG preparation is also a good source of the Hageman factor and suffices for the provision of an adequate and essentially unvarying amount of this factor in most of our test systems. The special problems encountered in testing for the Hageman factor will be discussed in the section dealing with that component.

Other reagents These too are appropriately described in the sections dealing with the specific factor testings for which special reagents are required. Generally factor deficient systems are prepared from human patients congenitally lacking the factor in question. Plasma eluates provide a suitable prothrombin (etc.) substrate. The supernate after the adsorption when used in suitable dilution e.g. 1:10, 1:15, 0.1 ml per 5 ml incubate is often a practical way to provide accessory factors (especially AHF) still maintaining the deficiency of the factor under consideration. Eluates heated to 52°C for 1/2-1 hour offer another means of securing deficiencies of such labile factors as V (AcG) or VIII (AHF) contaminant traces of which in fresh eluates may reduce the sensitivity of the specific tests.

SPECIFIC FACTOR ASSAYS In order to adapt the test system to the specific quantitative study of any single clotting factor it is necessary to obtain the system deficient in just the factor under consideration and then to study effects of that factor addition. In practice this is not difficult since it can be accomplished without the use of highly purified reagents. Moreover the deficiencies need not be absolute which would often be very hard to achieve. It is essential however to determine all the clotting factors in the test system and to ensure optimal or at least adequate and unvarying amounts of every one of them prior to addition of the factor to be tested. All this may require considerable preliminary experimentation in order to obtain suitable reagents and test conditions. The various methods including specific 1 stage tests which are described in the main thesis and elsewhere [534] are very helpful in these preliminary studies. The significant results made possible by these new refinements of the two stage method will be apparent in the data to follow.

PROTHROMBIN In the main thesis Experiment 10 (1956) and accompanying Table XII and Figure 14 illustrate use of the method in assay of prothrombin and Figures 10-12 show the excellent correlation of results in comparison with other methods. Another similar illustrative experiment (Experiment 51) was presented at the Montreux Meetings (August 1959) and its results are reproduced in Figures 21-22. Experiment 51 brings out one or two additional points. The data are for a BaSO₄ citrate eluate of bovine plasma and the preparation had been stored at 20°C for nearly three years. Complete absence of any detectible trace of thrombin was evidenced by failure to clot fibrinogen in several days in a control test. Excellent activation to thrombin however was obtained when the prothrombin containing eluate was activated by CaCl₂ AcG (BaCO₃-adsorbed beef serum) and human brain thromboplastin. Thus prothrombin and such accessory factors (VII = proconvertin; X = Stuart Prower factor) as must be provided in the eluate proved to be remarkably stable under the stated conditions of frozen storage.

The activation curves for the various eluate (prothrombin) dilutions shown in Figure 21 differ in shape, slope and position. These then indicate the activation rates which in practice are best measured by (a) the clotting time in the initial (1/2 or 1 min.) test and (b) the incubation period required to reach the optimum. The optimum is the shortest clotting time reached and this we use as a measure of

the thrombin yield as shown in Figure 22. Here the clotting-time optima from the same experimental data are plotted against the relative or "percentage" prothrombin concentrations (eluate dilutions). The higher the original prothrombin concentration the greater is the final thrombin yield that is the shorter the clotting-time optimum. A standard reference curve like this can be prepared for each substrate. In the case of the normal bovine plasma eluate in this particular experiment there seems to be a logarithmic linearity within a somewhat limited range of prothrombin concentrations as shown by the inset Figure 22. Pending investigation of conditions which may be required to produce this mathematically-satisfying result it is tentatively suggested that it may depend upon absence of inhibitors together with provision of optimal (or adequate) amounts of all factors other than the prothrombin. That prothrombin is the only significant variable may be deduced from the observation (Figure 21) that the optimal incubation periods were practically the same (5-6 min) in all these tests except at the 10% dilution. In this older experiment accessory factors (VII-X) were being diluted along with the prothrombin. Clearly this would seem to make no difference provided they are over about 20% of their original level. In our sensitive test system even traces of accessory factors may suffice for considerable thromboplastin- and thrombin-formation if the incubation period is sufficiently prolonged. Conversely activation rates are particularly sensitive to variations in concentrations of accessory factors particularly below a certain optimum (see later). Some of these considerations may need some further study in connection with the reaction kinetics of prothrombin activation.

ACTIVATORS We propose to classify the factors which participate in the activation of prothrombin to thrombin into three categories: (1) Ca-ions already covered in the thesis and concerning which we shall not say more than to re-emphasize their necessity for the test systems under present consideration; (2) 'thromboplastic' mechanisms about which we shall have some new experimental data in the following pages; and (3) 'accessory factors': it is here we would place the newer clotting factors discovered in recent years. These were reviewed in the dissertation as of 1956. The International Committee for the Standardization of the Nomenclature of Blood Clotting Factors was established in 1954 under the chairmanship of Dr. Irving S. Wright [549]. After very careful weighing of the evidence as presented by leading workers in the field at a series of conferences including that recently (August 1959) [550] held in Montreux, the Committee has now agreed upon the designation by Roman numerals of the following factors in the blood-clotting system:

- Factor I: fibrinogen
- Factor II: prothrombin
- Factor III: thromboplastin (tissue)
- Factor IV: calcium
- Factor V: proaccelerin labile factor plasma Ac-globulin (AcG) etc

(Factor VI is unassigned)

- Factor VII: proconvertin SPCA etc
Factor VIII: antihemophilic globulin AHF (antihemophilic factor)
plasma thromboplastic factor A etc
Factor IX: PTC (plasma thromboplastic component) plasma
thromboplastic factor B Christmas factor etc
Factor X Stuart Prower factor etc

Note that the differentiation between Stuart (factor X) and SPCA (factor VII) is now convincingly established. The same is true of factors VIII (AHF) and IX (PTC) but other hemophiloid factors still need further clarification. PTA (plasma thromboplastic component C etc) according to data reviewed at the Montreux Conference appears to react with Hageman factor on certain types of surfaces to yield an activator product. Until the nature of these components and reactions are more clearly understood their nomenclature will remain under consideration. Spaet's fourth component (plasma thromboplastic component D) [446] turned out to be merely factor IX complicated by an inhibitor problem as in our own first case of PTC-deficiency (see thesis). Tentative evidence of still newer clotting factors or possibly intermediates was also brought to the attention of the Montreux conference particularly by Dr Koller and by Dr Alexander. Our own contribution to the Montreux discussions was to present the following data on the study of the newer (and older) clotting factors by means of the versatile two stage method.

FACTOR VII Figure 23 shows the activation data obtained in a specific study of Factor VII (Experiment 52). The prothrombin substrate was a BaSO_4 -citrate eluate from oxalated plasma of a well studied congenitally hypoproconvertinemic young lady L B [535]. The usual activators namely Ca, AcG and tptn (human brain thromboplastin) were used for the data shown in the graphs. The top (0) curve is the control for the factor VII deficient system with no other additions than those just mentioned. It shows a slow activation quite satisfactory for testing effects of adding factor VII. As a simple source of factor VII we used the BaSO_4 citrate eluate from normal human serum. This of course contains some other accessory factors such as Stuart PTC etc but it was easy to show that the addition of these (as provided by a similar serum eluate from the VII deficient patient) did not change the control. Thus we could standardize our system for factor VII assay using the normal serum eluate in an amount of 0.1 ml per test as our 100 'per cent' empirical assay standard. This (100) and the 50, 10 and 2 per cent dilutions are illustrated in the chart clearly showing the assayability of factor VII by our two-stage method. Dr Alexander of course has performed similar studies on his original SPCA case material [521]. In agreeing with him we merely wish to stress one point which seems to be borne out in the present series of experiments. This is that the proconvertin here appears to affect only the reaction rate. Ultimate thrombin yields (or optimal C.T.'s) were the same in all tests including the control. We would not like to insist upon this point however unless we could be sure that the substrate was completely devoid of factor VII. Although this case was a severe bleeder with prothrombin times usually over 60 sec it is

not ruled out that she might still have only a relative deficiency of factor VII. Our system is very sensitive to even traces of accessory factors as noted above. The INSET TABLE (Figure 23) gives the actual test data which show the results almost equally well by simple inspection. Omitted from the curves but included in the table is a test of factor VII addition at the 200% level. This seems to be slightly inhibitory at the C T endpoint. Very much the same sort of thing occurs in our test system when almost any clotting is increased beyond a certain 'optimum'. As stated in the section on PROTHROMBIN the 1 min tests and the optimal incubation periods measure the activation rate, while the shortest clotting-time reached indicates the thrombin yield. In the present instance the similar optimal C T's clearly indicate complete or '100 per cent' thrombin yields in the control and with all factor VII additions up to its optimum.

The last two horizontal lines of the inset table record an important modification of our test system. This is to substitute cephalin for the usual tissue thromboplastin. Our routine 'cephalin' is the Bell Alton type of phosphatide extracted by chloroform from acetone-dried human brain as described earlier. We propose to term it a prothromboplastic phosphatide. Another current use of the term 'prothromboplastic' [545] for accessory (or co-)factors we consider to be invalid. Our use of the term indicates any phospholipid material which plays the role of an activator in the conversion of prothrombin to thrombin when it is provided with suitable cofactors in order to induce a certain type of reactivity in clotting systems. This type of r activity has been termed 'thromboplastin generation' [522] but some experiments (later) cause us to change the semantics of this idea somewhat. Tentatively going along with the 'thromboplastin generation' idea in the main thesis we stated that the above test modification (using ceph instead of tpin) converts our 2 stage system into a valuable new way to study such 'thromboplastin generation'. Of course it is necessary to have optimal (or adequate) amounts of all 'accessory' factors needed for thromboplastin generation. Of these most are provided relatively readily and the one that requires most careful control is AHF. Its adequacy is ensured in several ways. In the present case a factor VII free AHF was supplied by adding 0.1 ml of a 1:5 dilution of patient L B's supernatant plasma after BaSO₄ adsorption.

The extremely interesting point in this factor VII deficient system is that the absence (0) or presence (at optimal 100%) of factor VII makes no difference to the cephalin test results. Our tests confirm the conclusion which others [522] have previously arrived at by the thromboplastin generation test and other methods: namely that factor VII is not needed for thromboplastin generation.

HAGEMAN FACTOR Figure 24 shows data on the Hageman Factor (Experiment 53). Here the data are just the reverse of the factor VII results. The inset table shows that Hageman factor is without influence on prothrombin activation by tissue thromboplastin. However the curves clearly indicate that this factor is very much needed for the activation by cephalin: that is for the generation of thromboplastin. The control (0) without added Hageman factor (see below) was very poorly activated and successive increments 1, 10, 100 units of added Hageman gave a nice family of curves for Hageman factor.

In Experiment 53 the substrate was a BaSO_4 citrate eluate from oxalated plasma of one of our [534] markedly Hageman deficient cases (L G). This experiment presented some technical problems. Our ordinary AcG (BaCO_3 treated beef serum [492]) was found to contain Hageman but we [537] were able to remove it completely by continuous flow paper curtain electrophoresis at the expense of some loss in potency and stability of the AcG preparation. Dr R H Wagner [548] (of our Pathology Department) generously provided us with some of the purified AHF preparations which he had been fractionating from various animal bloods. The purified AHF used in the present experiments was quite satisfactory and devoid of Hageman according to the control (0) here and by all our usual tests. The Hageman preparation was one which we prepared from outdated blood bank plasma following the directions kindly supplied to us by Dr Ratnoff [543]. We have good data also on those fractions from our [534] paper curtain electrophoresis in which Hageman was the only clotting factor demonstrable.

STUART FACTOR Figure 25 shows data for the Stuart Factor [298 533] (Experiment 54). The substrate was a dialyzed $\text{Al}(\text{OH})_3$ phosphate eluate from citrated plasma of a severe congenital Stuart deficient (A G). The AHF was patient A G's Stuart free supernate after the plasma adsorption. The Stuart factor preparation was obtained from normal human plasma by continuous flow paper curtain electrophoresis by our associate Dr C L Johnston [534]. This was an excellent fraction rich in Stuart with no factor VII or any other clotting factor according to an exhaustive series of tests. It is now well known that Stuart factor differs from factor VII in being required for thromboplastin generation as well as for conversion of prothrombin to thrombin [533]. As expected therefore it turned out to be a significant variable in both types of our test system. Just a few of the activation curves are included in the composite chart of Figure 25. The initial activations in the Stuart deficient systems were often so slow that the ordinate in the top half of the chart was changed to minutes and the dotted lines indicate the break in the scale. These curves show the very poor activation in the two Stuart free controls with either type of thromboplastic agent. The thromboplastin curves are DASH lines and include just one with optimal 1:1 Stuart addition. A 1:10 and 1:1 Stuart addition are shown in the cephalin series. Particularly note that with optimal (1:1) Stuart the activation with cephalin is just as good as with thromboplastin (in fact the test times were point by point identical) except for the first minute. This last is an illustration of the initial delay in the phospholipid activated system which leads us to conclude that it takes a minute or two for (so called) 'thromboplastin generation' from the lipid to reach the equivalent of tissue thromboplastin in our test system.

OTHER ACCESSORY FACTORS Without encumbering this presentation with the experimental details we would like to state that we have good preliminary data analogous to the above for modified two stage assays of factor V factor VIII and factor IX. We have not yet had the opportunity to study a satisfactory case of PTA deficiency but believe this factor should be equally amenable to quantitation by the cephalin two stage method.

THROMBOPLASTIN FACTORS Figure 26 (Experiment 55) shows typical prothrombin activation curves using normal canine plasma eluate to test (I II) optimal cephalin or (III IV) optimal tissue thromboplastin respectively without or with factor VIII (AHF) all other factors being normal. The cephalin activation without AHF (I) is prolonged and deficient. With AHF (II) it is complete in a short time (3 min) but is still slow initially because of the delay required for 'thromboplastin generation'. The tpin activation is fast starting and rapidly completed (2-3 min) with no difference whether AHF is absent (III) or present (IV). Thus our two stage is a valuable way to distinguish between the two types of thromboplastic agents which illustrate what Dr Brinkhous and colleagues [282] designate by the operational terms 'partial' and 'complete' thromboplastins recognizing of course that both act only in conjunction with several other cofactors.

Table LXI summarizes results of similar tests (Experiment 56) on several thromboplastic materials all tested at their experimentally determined optimal concentrations with and without AHF. The percentage thrombin yield values are computed in the usual way. The rate data are replaced by qualitative descriptions which fairly indicate the basis for comparisons. Thus the alternatives 'fast' or 'slow' refer to the initial reaction rates while 'short' or 'long' refer to the optimal incubation periods. The differences between tissue thromboplastin (1) and cephalin (2) which were defined in Experiment 55 are restated. Test (3) is with Sbp, 'Inosithin' a crude alcohol-insoluble fraction of 'Asolectin' (soybean inositol phosphatide) [527 528]. *It gave evidence of contamination with some inhibitor. Test (4) is with Esp, a synthetic L- α -dioleoyl ethanolamine phosphatide recently prepared by Dr E Baer of Toronto. *its potency was weak but significant and it did better in another type of test system (with Stypven) which will be described later. Test (5) is with Plat, human washed platelets obtained with the ADL Cohn Fractionator [546] they probably retained a little AHF contaminant. Test (6) is with Rbc, human erythrocyte hemolysate prepared by Dr Quick's [532] method. Test (7) is the very satisfactory control. Clearly all these agents 3-6 behave like cephalin with significant improvement of their activator function on adding AHF. In this they are all unlike tissue thromboplastin and therefore fit the subcategory of partial thromboplastins or as we prefer to designate them prothromboplastic factors.

Experiment 57 deals with prothrombin (canine eluate) activations by (I) optimal cephalin (II) tissue thromboplastin at 1/10 optimal strength and (III) a mixture of I and II. AHF was added in series (A) and omitted in (B). The essential data are emphasized in the simplified charts of Figure 27 which merely indicate progression from the initial (1/2 min) clotting times to the optima shown details of the intervening curves being omitted. Clearly the mixtures III were better than with either thromboplastic agent alone. If we had merely done experiment (A) with adequate AHF we could have explained the result simply as the additive effect of extra thromboplastin 'generated' from the phospholipid. But the synergism was equally good in (B) without the AHF when the cephalin alone (B II) was very inadequate because of the deficiency in so called 'thromboplastin generation'. How then can we explain this result? Before attempting to answer this question we shall present another line of experimental enquiry.

STYPVEN The following experiments modify our two stage further by introducing the artificial additive Stypven (Russell's viper venom). The reason for this follows. We were reinvestigating the 2 stage method as a suitable procedure for assaying thromboplastin in the usual system composed of normal plasma eluate adequate in Stuart (X) and factor VII as well as prothrombin and with optimal added Ca and AcG various tissue thromboplastins can be compared with our standard human brain preparation which is a satisfactory standard although it dilutes out rather rapidly. Our cephalin likewise seemed to be a good practical standard for assaying prothromboplastins. For such assays however the system needs in addition to the above factors (but excepting factor VII) adequate and controlled amounts of AHF, PTC, PTA, Hageman ? etc. The most significant of these variables is AHF (factor VIII) which is difficult to prepare, preserve and standardize. In looking for a way to circumvent this we tried Stypven. This now resulted in a somewhat different bioassay system but one which fulfilled excellently the requirements for prothromboplastin assay as shown by the following experiments.

Experiment 58 is the new Stypven modified two stage using canine plasma eluate, optimal AcG and calcium and a series of dilution of brain cephalin (Ceph) as the standard. Dr Baer's synthetic dioleoyl cephalin (Eap) was the 'unknown' tested at two dilutions. Table LXII gives the results citing the clotting times after the 1 min. and the optimal incubation periods. Tests 1-7 are the standard cephalin dilutions and test 8 is the control. Tests 9 and 10 are the two 'unknowns' which check well and indicate the 1:1 Eap to be equivalent to about 3% the potency of the 'standard' cephalin. Without attempting to assess possible losses or inactive material but on a simple weight for weight basis the Eap potency was slightly less than 1/400th that of the Ceph. Although this is weak it is by no means inconsiderable. The preparation of 1,2-dimyristoyl phosphatidyl α -ethanolamine mentioned in the section on synthetic phosphatides in the main dissertation was also retested in the new system and found to be inert. It would appear that Dr Baer by introducing unsaturated fatty acids into the cephalin molecule has now achieved a genuine synthesis of a prothromboplastic phosphatide. It could be merely a matter of the best fatty acids and physico-chemical homogenization to give us a really potent preparation.

The demonstrable activity of brain cephalin at a dilution of 3 in 10,000,000 (test 7) may indicate a fact of great physiological significance namely that an extremely minute amount of prothromboplastic lipid can suffice. This may indeed be the normal 'trigger mechanism' for the initiation of blood clotting.

Experiment 59 was designed to explore the mode of action of Stypven by testing in a series of factor deficient incubates.

REAGENTS Prothrombin containing substrates (eluates) were from normal (dog) and human congenital deficiencies of proconvertin (factor VII) case L.B. of Stuart (factor X) case A.G. or of Hageman L.C. (see earlier). The PTC (factor IX) deficient was the Al(OH)₃ phosphate eluate from a severe case (A.Y.) of Christmas disease. AHF (factor VIII) and AcG (factor V) deficiencies were secured experimentally by heating the normal canine eluate to 52°C for one hour. The AcG AHF (purified) Stypven (Burroughs Wellcome) and throm

boplastic agents were the same as in the preceding experiments. Stuart (factor X) preparations were (A) the bovine "proconvertin" containing both Stuart and factor VII which is described in the main dissertation and (B) Stuart factor free from all other clotting factors purified by continuous flow paper curtain electrophoresis [534] as in Experiment 54. The Hageman factor preparation was described in Experiment 53.

TEST SERIES (I) Using normal (dog) eluate and optimal Ca cephalin and AcG the optimal concentration of Stypven was first determined. This was 0.2 ml of 1:10,000 venom solution per usual 5 ml incubate. This amount was used in all the subsequent experiments.

TEST SERIES (II) included the following additions to normal eluate: (1) Ca AcG AHF (no Stypven or cephalin); (2) Cephalin AcG AHF Stypven (NO Ca); (3) Ca Stypven (NO cephalin AcG or AHF); (4) Ca Stypven AcG (NO cephalin or AHF); (5) Ca Stypven AHF (NO cephalin or AcG); (6) Ca Stypven AcG AHF (NO cephalin). No significant thrombin formation occurred in any of these controls, thus proving the necessity for Ca ions and for prothromboplastic phosphatide. Incidentally they show also that the lipid must be extrinsic and not come from any of the protein materials tested.

TEST SERIES (III) is shown in Table LXIII recording the deficiencies, the additives, and the initial ($\frac{1}{2}$ -1 min) and optimal clotting test results. The summarized conclusion is that Stypven with cephalin and optimal Ca AcG and Stuart factor (adequate in all these eluates) gives rapid and complete thrombin formation even when the system is deficient in (1) AHF (2) PTC (3) Hageman or (5) factor VII. Since our usual bovine AcG (Experiment 53) contains Hageman factor, the supernatant BaSO_4 plasma (diluted 1:10) from L.C. was substituted in test 4 in order to supply AcG without factor X (Hageman). It worked almost as well as the usual AcG (test 3). The AcG-deficiency obtained by heating the normal canine eluate according to test 6 was not absolute but the Stypven test results were poor compared with the unheated (AcG+) AcG supplemented eluate in test 14. In test 7 Stypven was omitted and the hemophilic (AHF-) system showed the usual poor activation with Ca cephalin AcG. In tests 8-11 the various deficient eluates all gave very little thrombin formation when Stypven was used without cephalin. In test 12 with Stypven plus cephalin there was deficiency of AcG as well as Hageman. In test 13 a potent AcG free Hageman preparation (see Experiment 53) was added (Hag+) without improvement. This confirms the need for AcG in the Stypven-cephalin test system.

TEST SERIES (IV) was a special study of relationships of thrombin formation to factor X (Stuart), the substrate being eluate from case A.G. (Experiment 54) as noted under Reagents. All additions were optimal for the preparations used. Table LXIV records the experimental data including computations of the percentage thrombin yields. Stypven alone (test 1) and when Stuart factor was added (test 2) proved ineffective. With Stypven plus cephalin (test 3) the 52 per cent thrombin yield was inadequate compared to results with added Stuart factor preparation A, test 4 or B, test 5. Tissue thromboplastin showed its

usual (Experiment 54) inadequacy in the Stuart deficient system test 8 and adding Stypven test 6 did nothing to the thrombin yield but may have caused a slight improvement in the activation rate. Again restoration of Stuart factor test 7 returned this system to normality both as to rate and thrombin yield. This could also be accomplished without the Stypven tests 9-10 when either Stuart preparation was added. Cephalin with AHF (test 11) showed very slow and incomplete activation and this could be normalized by Stuart factor addition without any Stypven (test 12). Besides confirming previous (Experiment 54) two stage test findings on the Stuart factor these new results clearly indicate that Stuart factor is needed for the modified test system employing Stypven plus cephalin.

DISCUSSION These experiments show in the clearest possible manner that Russell's viper venom (Stypven) is not itself a thromboplastin nor prothromboplastin (by our definition) but is a special type of accessory factor acting in its own right. It is specifically accessory to prothromboplastic phosphatide (e.g. cephalin) and needs also Ca, AcG and Stuart factor in order to convert prothrombin into thrombin. Stypven does not act like factor VII or any other physiological accessory factor. The venom activates a factor VII deficient system not because it replaces proconvertin but simply because its reactions with the phosphatide and other activator components do not require factor VII. Only tissue thromboplastin needs factor VII and Stypven does not improve tissue thromboplastic activity significantly in our two stage test system as shown in the next experiment.

Experiment 60 These are our usual activations of normal eluate (canine) with optimal Ca and AcG but varying the concentration of brain tissue thromboplastin (100% = optimal) with or without the addition of optimal Stypven. The $\frac{1}{2}$ min and optimal clotting times are recorded in the simplified presentation of Figure 28 which emphasizes the essential findings. The addition of Stypven in no case changed the optimal clotting time or optimal incubation period to any significant extent. There may possibly have been a slight improvement in the initial ($\frac{1}{2}$ min) activation rates with the Stypven addition but the data are not sufficiently convincing on this minor point. All in all we fail to find any real evidence that Stypven works with tissue thromboplastin.

Experiment 61 was designed to show that the other prothromboplastins which so clearly resembled brain cephalin in the tests of Experiment 56 could all substitute for the routine lipid in the Stypven modified two stage system. The data given in Table LXV are sufficiently explanatory it being quite evident that all the tested 'prothromboplastic' preparations gave excellent and similar (final) thrombin yields in this test system. Even the synthetic Eap was very satisfactory when the incubation period was sufficiently prolonged.

TRYPSIN and THROMBIN Further diversity of activator systems can be shown experimentally. We have some good preliminary data to demonstrate that trypsin or a trace of thrombin can act as accessory factors in our cephalin modified two stage system. These agents act only in the presence of Ca ions and prothromboplastic lipid (cephalin). They also require a group of other cofactors including V

VII VIII IX and X but not Hageman in the thromboplastin and thrombin-forming incubation systems

FINAL INTEGRATION OF IDEAS

These new experimental data definitely contribute further evidence in support of our major thesis concerning the key role of prothromboplastic phosphatide (probably cephalin) in the early phases of prothrombin activation. The facts (a) that tissue thromboplastin, certain cephalins and a variety of preparations known to be rich in phosphatides all serve the common purpose of assisting in the activation of prothrombin to thrombin together with (b) the significant diversity of the groups of 'accessory' factors which can work with them to achieve this result particularly in the prothromboplastic test systems seem to us to require a fundamental explanation. This we suggest should not be in terms of factor entities as such but rather in terms of the dynamic processes involved. In order to emphasize these processes and the common features of diversely constituted test systems we suggest that the substantive term thromboplastin be avoided and replaced by the dynamic concept more appropriately conveyed by the adjectival thromboplastic mechanisms. The further descriptive characterization prothromboplastic applicable to the role of certain phosphatides (e.g. cephalin) seems fully justified by the experimental evidence. Our main thesis defends the working hypothesis that the 'availability' of such prothromboplastic factors constitutes 'the trigger mechanism' and determines the subsequent series of intermediary reactions essential for the formation of active thrombin and hence for blood clotting. In recent years the concept of 'thromboplastin generation' has gained wide acceptance. It may seem apparent to many readers that we have been talking around the subject of 'thromboplastin generation' and that as we ourselves have indicated our new uses of the two stage test may be regarded merely as another way to demonstrate the phenomena of this so-called thromboplastin or 'intrinsic' thromboplastin generation. It may be a question of semantics but we believe that some of our data require a different concept. It is particularly difficult to explain Experiment 57 on the synergistic action of cephalin with suboptimal tissue thromboplastin in the absence of factors essential for the recognized 'thromboplastin generation'. Then again the 'intrinsic' thromboplastin shows differences from 'extrinsic' (tissue) thromboplastin in such things as factor VII need and in significance for clinical hemostasis the complete answers to which have not yet been worked out. We propose therefore a new clotting theory based on the ideas of our main thesis but carrying the concepts somewhat further. We suggest the hypothesis of phospholipid transfer mechanisms as basic to the coagulation reactions. This working hypothesis envisions prothromboplastic phosphatide made available at the very outset of the blood changes that lead to clotting. Whether this be primarily from platelet alterations (fully discussed in the dissertation) from surface-activated plasma factors (bringing in the recently discovered interactions involving Hageman factor and PTA) [550] or biochemical changes in certain lipids and plasma lipoproteins (as the very recent work of Dr. Esnouff [524] is beginning to suggest) and what it is that coordinates these processes need further

elucidation. Many accessory factors are clearly involved and there must be an ordered sequence of intermediary reactions. It does seem reasonable to postulate phospholipid changes and transfers to a series of proteins through such a series of intermediate reactions. Pending the positive identification and perhaps isolation of such postulated intermediates we propose to adhere to a dynamic concept visualizing (a) trigger release of prothromboplastin lipid (b) a series of lipid and lipoprotein reactions (lipid transfer ?) and (c) final entry of prothrombin into these reactions with resulting alteration of its protein structure to yield the active enzyme thrombin. We have discussed at some length in the thesis the possible significance of certain proteolytic phenomena. What may turn out to be of more fundamental significance is the now demonstrated polypeptide-ester (e.g. TAME) splitting activities possessed in common by thrombin, Stypven [547], trypsin and fibrinolysin (= plasmin). An unexplored field until most recently is the possible relation of lipid altering enzymes (lecithinase ?) to coagulation and it will be interesting to watch for these developments in the future.

While many fundamental problems have still to be solved in the complex field of blood coagulation, our dissertation will conclude with a note of optimism. Much has been learned in the past and greatly added to in recent years. Rationalization of the majority of clinical problems has been advanced by the discoveries of the newer clotting factors. Practical tests for these are now available and as techniques are improved, factor isolation should become increasingly practical and hopefully of therapeutic application. The theoretical goal of basic understanding may be more difficult but many researchers are contributing toward it. Among the vast literature accumulating in this field, due place may perhaps be found for the contents of this compendium of experiments and thinking in the achievement of which the author's debt to his colleagues and associates is herewith gratefully acknowledged. Let us paraphrase the words of Robert Louis Stevenson and conclude: 'To travel together joyfully is better than to arrive' and the true success is to labour.

APPENDIX I

FIGURES AND ILLUSTRATIONS

FIGURES AND ILLUSTRATIONS

FIGURE	<u>Ref</u> <u>Page</u>
1 Skeleton scheme of basic reactions of blood clotting	15
2 Scheme of blood clotting and hemostatic mechanisms	16
3 The influence of varying thrombin concentration upon (a) clotting time (C T) and (b) fibrin yield (%)	17
4 Reaction kinetics of fibrin formation Four experiments with varying strengths of thrombin (0.1 0.2 0.3 0.4 units/ml) Residual fibrinogen as percent of original clottable protein plotted logarithmically after removal of clot at stated time intervals	17
5 The 'inverse law': clotting of fibrinogen with varying strengths (percentage relative) of thrombin plotted (a) directly (b) inversely	18
6 Dark field microscopy of clots formed from fibrinogen 'sol' by the action of (1) thrombin (2) papain (crystalline) (3 4) ninhydrin	22
7 A case (R J) of PTC deficiency with circulating anti coagulant (anti PTC) Clotting times of various recal cified plasma mixtures (normal + patient) in studies between February 29 and July 8 1952	28
8 Dark field microscopic observation of erythroplastid formation from a nucleated erythroblast from the bone marrow of a case of Hodgkin's disease	34
9 Correlation graphs of plasma analyses: 'prothrombin time' vs method II (orthodox 2 stage prothrombin assay)	57
10 Correlation graph of plasma analyses method II vs method I (specific 1 stage prothrombin assay)	57
11 Correlation graph of plasma analyses: method I vs method III ('eluate' 2 stage prothrombin assay)	57
12 Correlation graph of plasma analyses method III vs method II	60
13 Reciprocal clotting times (1/C T) for serial thrombin dilutions with three fibrinogens (I II III) decreasingly contaminated with 'serum factor' of L6rand	64

- 14 Minimal clotting-time 'end points' of suitably activated thrombin forming mixtures as a measure of relative concentration of prothrombin Method III 2-stage ('eluate') technique 76
- 15 Progressive inactivation of 'intermediary' thrombin by excess of oxalate (III) compared with absence of oxalate effect on fully-formed thrombin (IV) Activation of Seegers' purified prothrombin with added CaCl_2 and weak tissue thromboplastin Control tests: (I) in absence of oxalate and (II) with oxalate added only to the test fibrinogen 83
- 16 Reference curves for assays (on dog plasma) of (a) prothrombin times (reciprocally); (b) prothrombin (specific); (c) proconvertin (specific); (d) proaccelerin (specific) One-stage technique; percentage dilutions plotted against clotting-times 91
- 17 Thrombin yields percentage from highly purified prothrombin incubated with: (I) Ca ; (II) Ca + trypsin; (III) Ca + thromboplastin (weak); (IV) Ca + thromboplastin + trypsin Dotted line indicates reference curve i.e. reciprocal clotting times (1000/C T) plotted against prothrombin dilution (percent) 105
- 18 Dark field microscopy of platelet alterations:
- 1 'Stellate' forms of platelets in citrated rabbit plasma 124
 - 2 Recalcified rabbit plasma showing (a) 'vesicular' alterations of platelets (b) fibrin filaments 127
 - 3 Coagulating human blood drop showing (a) platelet 'granulomere' and vesicular alterations (b) erythrocytes (c) neutrophil granulocyte (d) a few fibrin needles 124
 - 4 Saponin lysis of platelets in citrated rabbit plasma 127
- 19 Dark field microscopy of megakaryocytes and thrombocytes
- 5 Megakaryocyte from rabbit bone marrow in autologous serum showing granular vesicular 'excrescences' at periphery 127
 - 6 Megakaryocyte from rabbit bone marrow recalcified after citration showing 'vesicles' at periphery 127
 - 7 Disintegrated thrombocyte in frog blood showing 'vesicles' The thrombocyte is lying on a large fibrin thread 128
 - 8 Recalcified citrated blood of fresh water sun fish showing (a) disintegrated thrombocytes (near center of photomicrograph) with vesicles and granules scattered by the explosive disruption of the thrombocyte (b) many nucleated red cells 128

FIGURES AND ILLUSTRATIONS 167

20	Dark field microscopy of erythrocyte alterations and of myelin figure formation from lecithin:	
9	Primitive erythroblasts from heart blood of rabbit embryo showing stromatolytic filaments	128
10	Erythroblast of tadpole showing numerous filamentous stromatolytic forms	128
11	Human erythrocytes showing contact development of envelope (membrane) filaments	128
12A	Edge of lecithin film (lipid air interface) showing smooth refractive edge	129
12B	Same edge showing 'myelin forms' produced by contact with distilled water	129
21	Prothrombin activation 26°C; varying prothrombin Two stage method	151
22	Prothrombin yields optimal clotting times varying prothrombin (eluate) concentration	152
23	Prothrombin activation 25°C; varying factor VII (proconvertin)	153
24	Prothrombin activation 25°C; varying Hageman factor	154
25	Prothrombin activation 25°C; varying factor X (Stuart)	155
26	Prothrombin activation curve : two stage method Cephalin <u>versus</u> tissue thromboplastin	156
27	Prothrombin activation: two-stage method Activation by I. thromboplastin 1:10 II cephalin 1:1 (optimal) III: I + II; (A) with AHF (B) without AHF	156
28	Prothrombin activation: two-stage method with and without Stypven; varying thromboplastin	159

Figure 1 SKELETON SCHEME OF CLOTTING MECHANISM

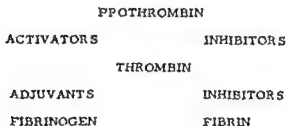
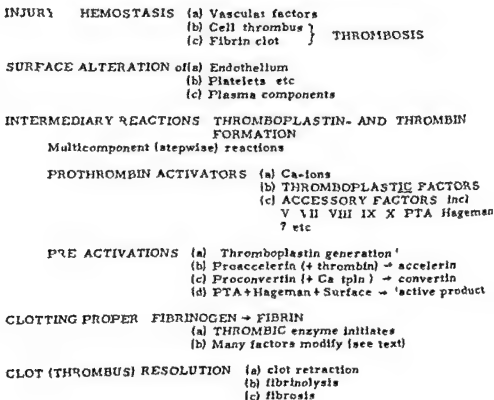


Figure 2 HEMOSTATIC AND CLOTTING MECHANISMS



EFFECTS OF CHANGE IN THROMBIN CONCENTRATION ON THE SECOND PHASE OF CLOTTING

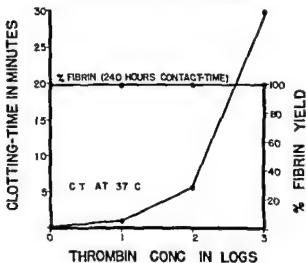


FIGURE 3

Figure 1 SKELETON SCHEME OF CLOTTING MECHANISM

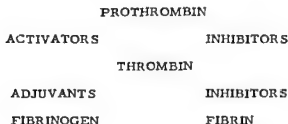
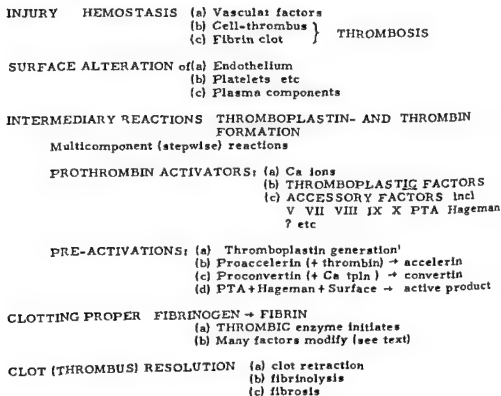
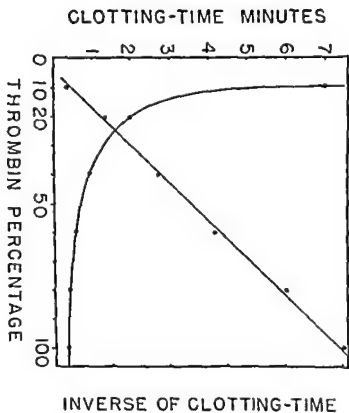


Figure 2 HEMOSTATIC AND CLOTTING MECHANISMS





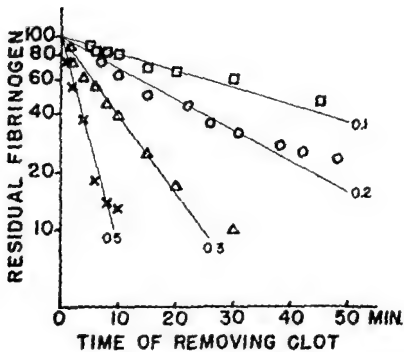
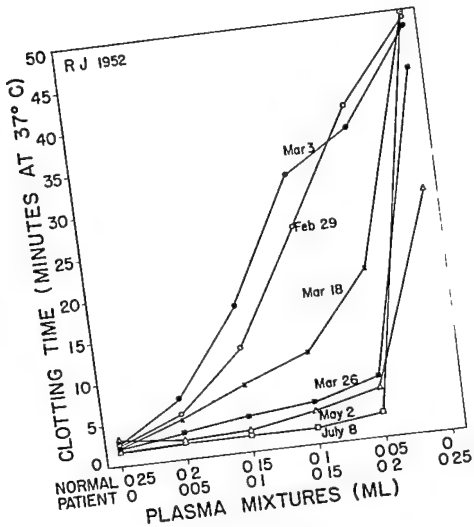


FIGURE 4



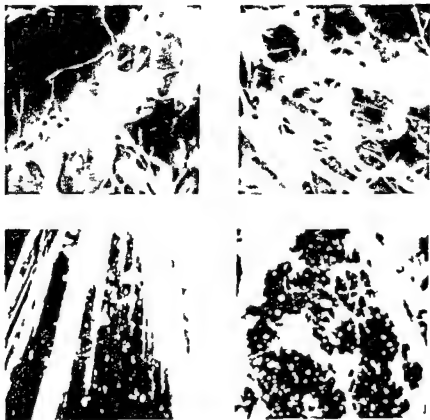


FIGURE 6

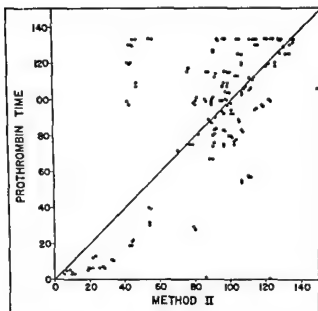
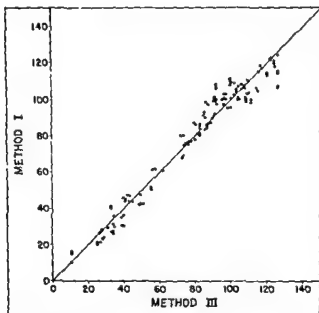




FIGURE 8



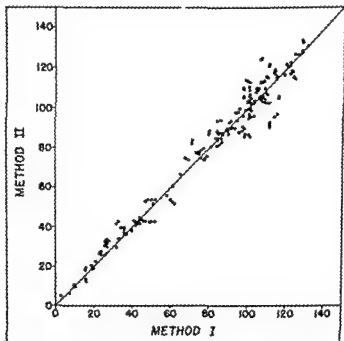
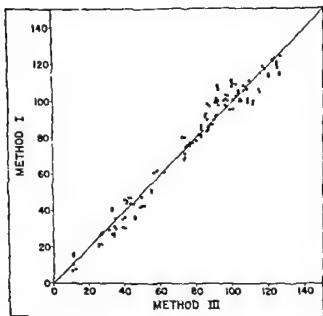


FIGURE 10



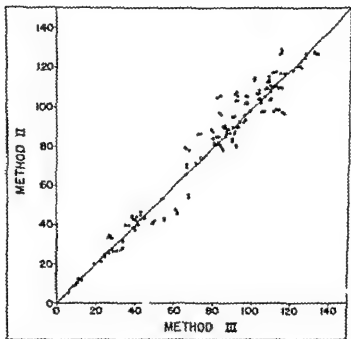
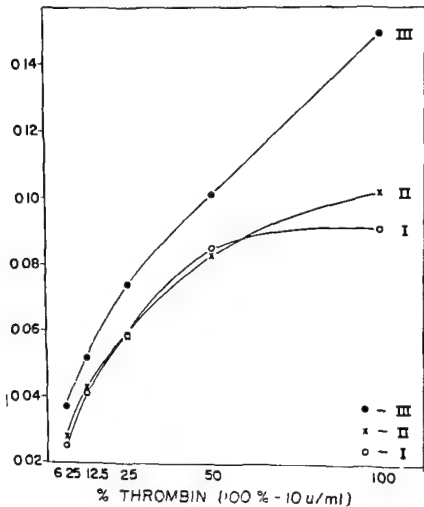


FIGURE 12

$\frac{1}{CT}$ AT VARYING THROMBIN CONCENTRATIONS



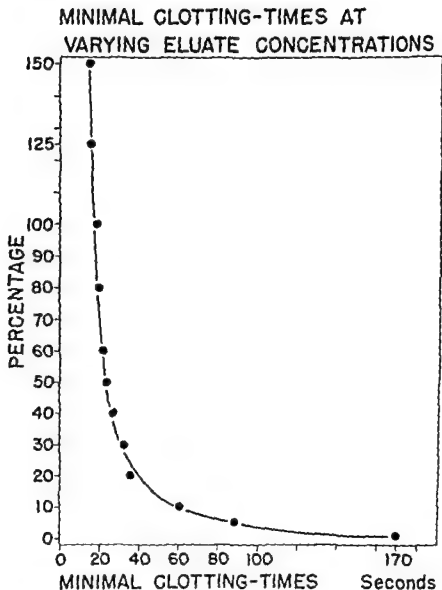
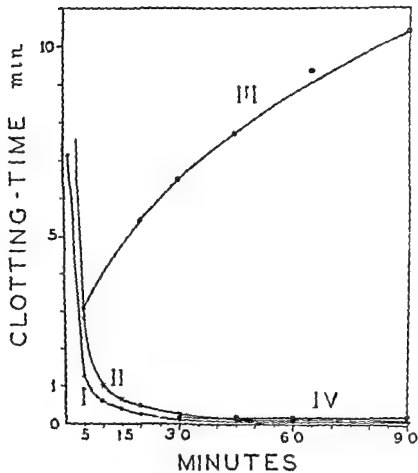


FIGURE 14

OXALATION



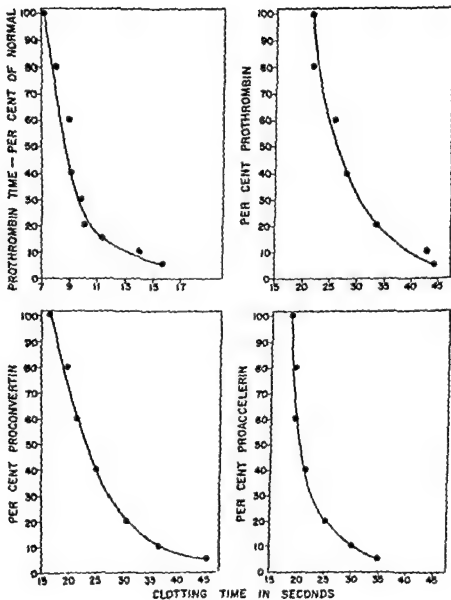
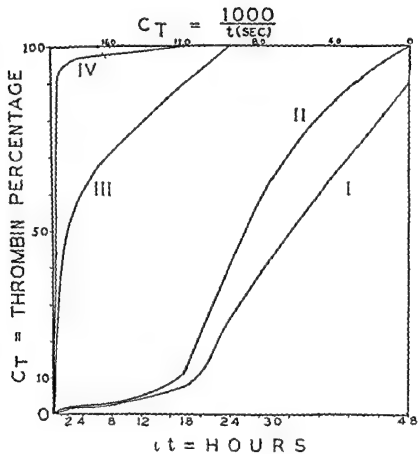


FIGURE 16



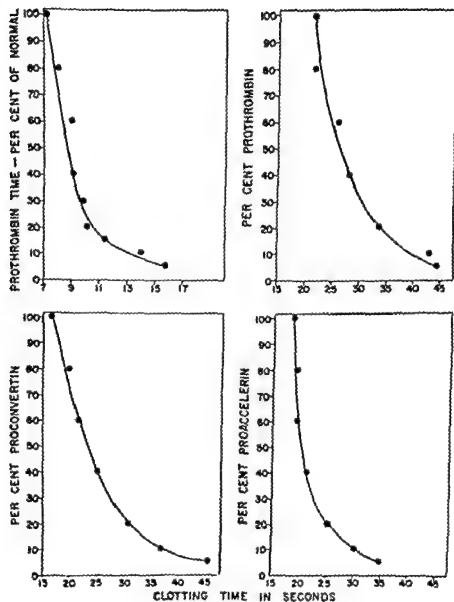


FIGURE 16



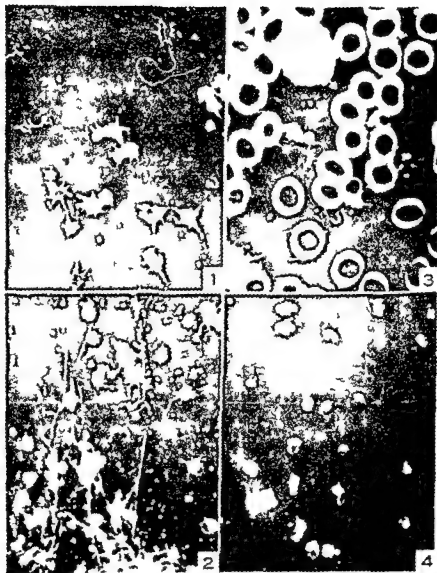


FIGURE 10

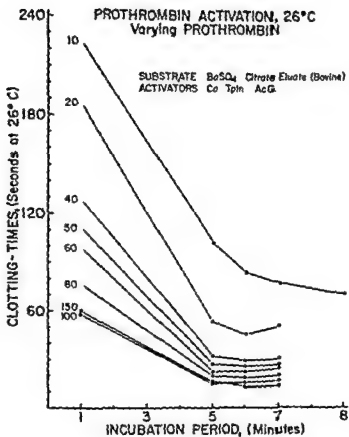


FIGURE 21

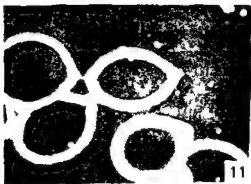
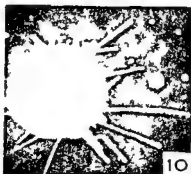


FIGURE 20

PROTHROMBIN ACTIVATION 25 C.
Varying FACTOR VII (PROCONVERTIN)

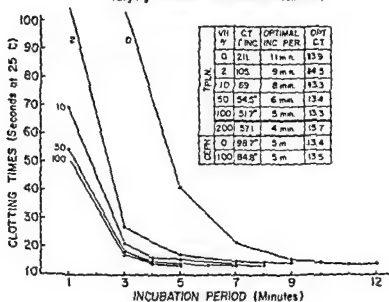
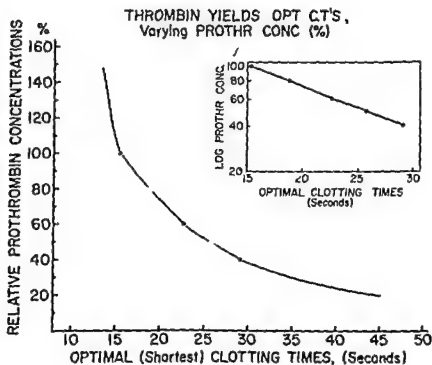


FIGURE 23



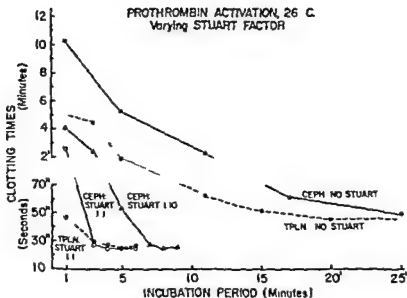
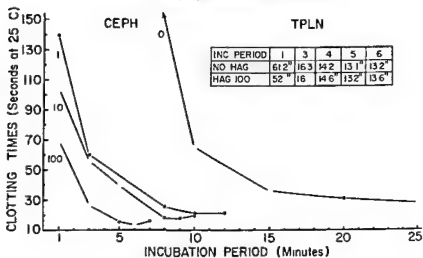


FIGURE 25

PROTHROMBIN ACTIVATION 25 C
Varying HAGEMAN FACTOR



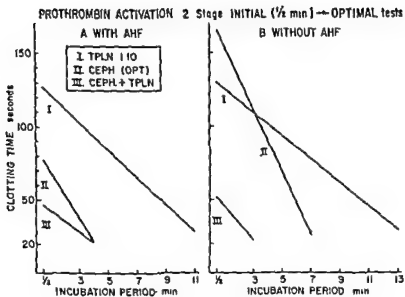
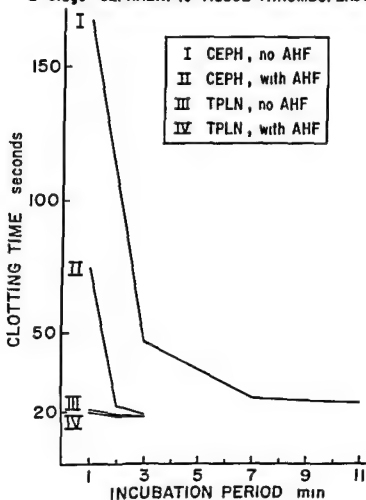


FIGURE 27

PROTHROMBIN ACTIVATION CURVES
2-Stage CEPHALIN vs TISSUE THROMBOPLASTIN



APPENDIX II

TABLES OF EXPERIMENTAL DATA

PROTHROMBIN ACTIVATION 2-Stage
INITIAL ($\frac{1}{2}$ min) \rightarrow OPTIMAL tests

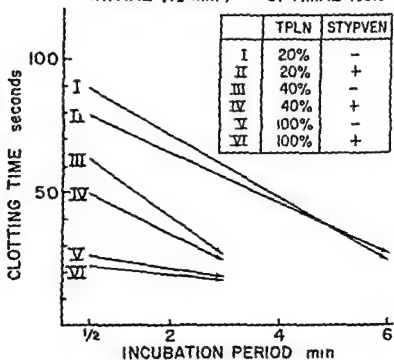


TABLE I Clotting of Salt treated Mixtures on Dilution after Stated Incubation Periods Seconds 24°C

	MIXTURE + 4.5% NaCl	ADDED with water	INCUBATION PERIOD (minutes)				
			1/2	5	10	15	20
1	Thr + Fibr	-	150	85	15	5	
2	Thrombin	Fibrinogen	190	200	205	215	2
3	Fibrinogen	Thrombin	240	245	250	330	295

* Mixture (1) clotted in 18 3/4 min

TABLE II Clotting times with Varying Amounts of (T) Thrombin of (F) Fibrinogens (I II III see text) Containing Different Amounts of Serum Factor

0.2 ml F + 0.2 ml T (bovine Upjohn's at [final] unitage stated) seconds at 25°C

F	10	5	2.5	1.25	0.625 (T units/ml)
I	11.2	11.8	17.0	24.4	39.2
II	9.8	12.1	16.9	23.3	35.8
III	6.7	9.9	13.5	19.1	26.9

TABLE III Clotting times with Varying Amounts of Calcium of Mixtures of Thrombin and Fibrinogen Containing Different Amounts of Serum Factor

0.2 ml F + 0.1 ml CaCl₂ ([final] molarity stated) + 0.1 T (5 units/ml) seconds at 25°C

F	0.02	0.01	0.005	0.002	0.001	O(H ₂ O) Ca(mol)
I	18.2	10.9	9.7	9.8	10.3	12.6
II	22.2	10.8	9.6	9.7	10.3	12.6
III	15.5	10.7	9.0	5	9.8	12.6

TABLE VI Specific Assays of Eluates

Unitage per ml expressed as percentage of standard normal human plasma values (100 percent mean) PTC and AHF assays are given in Table XXXVII B = benzene extracted (see text)

	ELUATE (modified)	PROTHR (2 stage)	PROCONV (1 stage)	PROACCEL (1 stage)	THR	PTC	AHF
a	Untreated	183	100	u	0		
b	Dialyzed	167	100	trace	0	+	+
c	Dial (b e)	171	115	trace	0		
d	B e (only)	183	105	1	0		

TABLE VII Test for Antithrombin in Dialyzed Eluate and in Serum AcG Preparation both reagents benzene extracted

INCUBATION PERIOD (minutes)		14	5	10	15	20	30
1	Saline control	26.1	26.9	24.6		24.0	23.9
2	Eluate	34.4	34.7	35.7		36.4	38.8
3	AcG (I)	23.3	33.0	128.4	195.0	267.0	
4	AcG †	22.0	25.8	29.9	31.6	35.1	42.8

Clotting times (sec) for 0.2 ml fibrinogen + 0.2 ml samples of thrombic mixture (T.M.) kept in siliconed tubes and tested (in glass) after the stated incubation periods T.M. = 1.0 ml thrombin (bovine Upjohn's 20 units/ml) + 1.0 ml of (1) saline (2) eluate; or (3) AcG (I) untreated (4) AcG † partly purified (see Experiment 10 p. 75)

TABLE IV Clotting of Platelet free (Dog) Plasma Effects of Glass (G) vs Silicone (S) Surfaces and Thromboplastic Agents viz (1) Platelets (A) Intact (B) Disintegrated and (2) Cephalin Clotting times seconds at 38°C after optimal recalcification

ADDED	SALINE		PLATFLETS (A)		PLATELETS (B)		CEPHALIN	
SURFACE	S	G	S	G	S	G	S	G
S	∞	∞	435	170	465	185	155	60
*S	∞	∞	550	195	660	235	150	60
*G+	∞	∞	180	170	210	205	30	30

*Held at 34°C for 1 hr with silicone (S) or powdered glass (G+)

TABLE V Clotting of Platelet free (Dog) Plasma Optimally Recalcified with Additives Noted Seconds 38°C

	ADDITIVE	CLOTTING TIME
1	Saline	∞
5	Soluplastin	7
6	Cryst trypsin (100)	118
7	Fibrinolysin (4 mg)	127

TABLE IX A Clotting Times (seconds) at 28°C for Mixtures Containing Dialyzed Eluate which had been Extracted with Benzene before Dialysis 5 Days Previously

SAL	ELUATE	AcG b c	EXTR	CEPH 0.5%	FIBR b c	Ca 0.02M	CLOTTING TIME
1	0.1	0.1			0.4	0.3	207.6
2	0.2	0.1			0.4	0.3	340.0
3	0.1	0.1	0.1		0.4	0.3	280.6
4	0.1	0.1		0.1	0.4	0.3	218.6
5	0.1	0.1		0.1	0.4	0.3	57.6

B Clotting Times of Above Eluate at Various Periods After Re extraction with Benzene

					5	15	3 hr
					min		
1	0.1	0.1	0.1		0.4	0.3	243.4 (443.5)
2	0.2	0.1			0.4	0.3	721.0 509.6
3	0.1	0.1	0.1		0.4	0.3	483.1 273.5
4	0.1	0.1		0.1	0.4	0.3	230.3 180.0
5		0.1	0.1	0.1	0.4	0.3	69.9 36 (6 hr)

TABLE VIII Clotting Test Mixtures (ml) of Fibrinogen (b e) and Dialyzed Eluates (see text) Effects of Benzene Extraction

Clotting times seconds at temperature stated

TEST	SAL	ELUATE	AcG (b e)	CEPH (0.5%)	FIBR (b e)	Ca (0.02M)	CLOT TIME (26°C)
1	0.2	-	0.1		0.4	0.3	475
2	0.1		0.1	0.1	0.4	0.3	450
3	0.4	0.1 (b e)	0.1		0.4	-	2250+
4	0.3	0.1 (b e)	0.1	0.1	0.4		2340
5	0.2	0.1 (b e)	-		0.4	0.3	1005
6	0.1	0.1 (b e)	-	0.1	0.4	0.3	221
7	0.1	0.1 (b e)	0.1		0.4	0.3	210
8		0.1 (b e)	0.1	0.1	0.4	0.3	69.6
9	0.2	0.1 (untr)			0.4	0.3	354
10	0.1	0.1 (untr)		0.1	0.4	0.3	151
11	0.1	0.1 (untr)	0.1		0.4	0.3	107.5
12		0.1 (untr)	0.1	0.1	0.4	0.3	46.3

SUPPLEMENTARY TESTS substituting tissue thromboplastin* for the cephalin (37°C)

13		0.1 (b e)	0.1	0.1*	0.4	0.3	16.7
14		0.1 (untr)	0.1	0.1*	0.4	0.3	15.7

TABLE XI Thrombin Formation from Undialyzed Eluates with Varying Activators

A Components of Thrombic Mixtures (ml)

THR MIXT	SAL	IMID BUFF	ELUATE	PRO C	AcG 1 8	CEPH 0 5"	TPLN	CaCl ₂ 0 15M
4	3 9	0 5			0 1 (b e)			0 5
5	3 8	0 5	0 1 (b e)		0 1 (b e)			0 5
6	3 7	0 5	0 1 (b e)		0 1 (b e)	0 1		0 5
7	3 8	0 5	0 1 (b e)		0 1			0 5
8	3 7	0 5	0 1 (b e)		0 1		0 1	0 5
9	3 6	0 5	0 1 (b e)	0 1	0 1		0 1	0 5
10	3 7	0 5	0 1		0 1		0 1	0 5
11	3 6	0 5	0 1	0 1	0 1		0 1	0 5

B Clotting Times (seconds) for 0 2 ml fibrinogen + 0 2 ml thr mixt tested after stated incubation periods 28°C

TEST	MINUTES							
	1	2	3	5	7	10	30	60 90
4	1830					1346	932	750" 733
5	538					480		290 259"
6	84	43 8	34 2	21 4	18 3	21 7		
7	895"			450		216	177	181
8	21	16 3	19	20			-	
9	23 3	18 8		20 8				
10	21 5	20 8	18 3	21 3				
11	19 8	17 6	21 6"	21 7				

TABLE X Thrombin Formation from Benzene-Extracted Dialyzed Eluate
A Components of Thrombic Mixtures (ml)

THR MIXT	IMID BUFF SALINE	ELUATE be dial	AcG (BaSO ₄ ads) be (x 2) 1 8	EXTRACTIVE from plasma	CaCl ₂ (0.1M)
1	4 6	0 1			0 3
2	4 5	0 1	-	0 1	0 3
3	4 4	0 1	0 1	0 1	0 3

B Clotting Times (sec) first visible fibrin for 0.2 ml fibrinogen +
0.2 ml Thr Mixt tested after incubation periods stated 28°C

TEST	1 min	5 min	10 min	20 min	30 min	1 hr	2 hr	3 hr	48 hr
1	1170		1230	745	720	720	710	700	745
2	676		488	503	435	395	334	335	365
3	480	347	336	259	259	243	249	262	560

TABLE XIII Effects of Purified Antithrombin Globulin (AHG) on Thrombin Formation from Eluate

The eluate (see text) provides prothrombin proconvertin PTC (etc ?) AcG Ca and the stated THROMBO PLASTIC AGENTS are added (+) with or (0) without AHG Method III procedure at 27°C

Clotting times (seconds) for 0.2 ml fibrinogen + 0.2 ml thrombic mixture (T M) after stated incubation periods

T M	AHG	THROMBOPL AGENT	1	INCUBATION PERIOD (minutes)									
			2	3	5	7	10	15	20	30	60	100	1000
1	+	O	506		309		202		175 ^u	110	100	111	
2	+	Solupl 1/1	19.9	16.8	<u>15.9</u>	16.6		16.9					
3	O	Solupl 1/1	22.2	17.6	<u>15.0</u>	15.9	15.9	16.9					
4	+	Solupl 1/5	81.6	31.7	22.7	19.6	17.5	<u>17.1</u>	18.1				
5	O	Solupl 1/5	72.4	41.5	25.1	18	17.3	<u>15.4</u>	17.6				
6	+	Ceph 0.1%	233	32.3	16.8	<u>15.8</u>	<u>15.7</u>	16	16.2	18.6			
7	O	Ceph 0.1%	141	104	69.6	40.1	34	28 ^u	<u>25.5</u>	26.9			

TABLE XIII Effects of Purified Antithrombophilic Globulin (AHG) on Thrombin Formation from Eluate

The eluate (see text) provides prothrombin proconvertin PTC (etc ?) AcG Ca and the stated THROMBO PLASTIC AGENTS are added (+) with or (0) without AHG Method III procedure at 27°C

Clotting times (seconds) for 0.2 ml fibrinogen + 0.2 ml thrombic mixture (T M) after stated incubation periods

T M	AHG	THROMBOPL AGENT	INCUBATION PERIOD (minute)															
			1	2	3	5	7	10	15	20	30	60	100	111				
1	+	O	506			309		202		175		100	111					
2	+	Solupl 1/1	199	168	<u>15.9</u>	166			169									
3	O	Solupl 1/1	2.2	176	<u>15.9</u>	159	159	169										
4	+	Solupl 1/5	816	317	227	196	175	<u>17.1</u>	181				-					
5	O	Solupl 1/5	724	415	251	18	173	<u>15.4</u>	176				-					
6	+	Ceph 0 1"	233	323	168	<u>15.8</u>	<u>15.7</u>	16		162	186							
7	O	Ceph 0 1"	141	104	696	401	34	28	<u>25.5</u>	269								

TABLE XIV Effects of Sequestrene Decalcification on Thrombic Activity of Prothrombin + Activator Mixtures Tested at Different Times from Start of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) for tests at stated periods timed from start of experiment

- (A) T M incubated alone and EDTA added to fibrinogen
 (B) T M incubated with EDTA from 5th minute
 (C) T M incubated with EDTA from 68th minute

TEST SERIES	TIME OF TEST (minutes) from start of incubation of original thrombic mixture (A)									
	2	4	5	10	15	30	60	68	78	128
A	3600+	609	297	86 9	78 2	85	97			
B			312	397	-	620	1440+			
C								74	73 5	73

TABLE XV Sequestrene Reversal of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) for tests at stated periods from start of experiment (A B) or from start of incubation (C D)

MIXTURE	INCUBATION PERIOD (minutes)									
	1/4	1	2	3	5	10	30	60	90	185
(A) T M (alone)			80.3		44.6	47.8	46.7	45.1 ^m	46.4	47
(B) T M + EDTA			108.4		117	135	152.4	174	195	209 ^m
(C) Dialyzed (B)	189	184	186	181	183	192	211			
(D) T M (alone)		146	34.6	25.6	25.5 ^m	26				

TABLE XIV Effects of Sequestrene Decalcification on Thrombic Activity of Prothrombin + Activator Mixtures Tested at Different Times from Start of Thrombin Formation

Two stage method at 28°C as described in text Clotting times (seconds) for tests at stated periods timed from start of experiment

(A) T M incubated alone and EDTA added to fibrinogen

(B) T M incubated with EDTA from 5th minute

(C) T M incubated with EDTA from 68th minute

TEST SERIES	TIME OF TEST (minutes) from start of incubation of original thrombic mixture (A)									
	2	4	5	10	15	30	60	68	78	128
A	3600+	609	297'	86 9	78 2	85	97			
B			312'	397'		620	1440+			
C								74	73 5	73

TABLE XVIII Effects of Treatment with Dowex '50' Upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 28°C for 0.2 ml (or equivalent) test mixture + 0.2 ml 1% Bovine Fibrinogen after stated periods subsequent to adding agent cited

TEST SERIES	AGE OF T M	ADDED AGENT	INCUBATION PERIOD (minutes)							
			1	3	5	10	15	20	30	60
A		Activ Mix	+	1320	217.5	33.5	26.5	29		37
B	10 m (A)	Dowex '50'	44.9		150.6	380		496	510	
C	60 m (A)	Dowex '50'	56.2		98.1	125.3		141.8	152.3	-
D	20 m (B)	Activ Mix	213		<u>203.6</u>	254.5		321.6	-	
*E		Activ Mix			26'	25"	<u>22.8</u>	23.2	26.2	

* Original eluate treated for 1 hr with Dowex '50' (next day)

TABLE XVI Effects of Incubation with Oxalate upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 38°C for 1 ml test mixture + 1 ml fibrinogen after stated periods of incubation subsequent to dilution with agent stated

TEST	AGE of T M	ADDED AGENT	INCUBATION PERIOD (minutes)						
			1/4	5	15	30	60	90	120
A	11 m	water	15	15	15	15	15	15	20
B	12 m	oxalate	55	95	160	390	1380	2700	4500
C	4 hr	oxalate	40	40	45	40	60	50	50

TABLE XVII Effects of Incubation with Citrate upon the Clotting Times of Various Thrombic Mixtures (see text)

Clotting times (seconds) at 38°C for 1 ml test mixture + 1 ml fibrinogen after stated periods of incubation subsequent to dilution with agent stated

TEST	AGE of T M	ADDED AGENT	INCUBATION PERIOD (minutes) ¹						
			1/4	5	15	30	60	90	120
D	9½ m	water	15	10	15	15	15	15	20
E	10 m	citrate	105	195	210	270	765	840	
F	3¼ hr	citrate	215	150	140	145	145		145

TABLE XXI Rates and Yields (Minimal Clotting Times) of Thrombin in Standard Thrombic Mixtures (T M) with Varying Concentrations of Cephalin

Ceph conc (final) in micrograms (γ) per ml of T M Clotting times (seconds) at 28°C for 0.2 ml fibrinogen + 0.2 ml T M tested at the stated incubation periods → denotes tests not carried to completion

T M	CEPH CONC, γ	INCUBATION PERIOD (minutes.)								FINAL† THROMBIN	
		5	10	15	20	30	35	40	45	→	→
0	0	502	462	36 _w	334	315 ^w			245 ^w		trace
1	100	77.2	38.3	29.1	31.3	31.5					38 ^w
2	50	52.2	26.3	25	22	23.3	27				58 ^w
3	25	54	44	30	26.9	40					40 ^w
4	12.5	67.3 ^w	46.9	41.6	38.5	31		30		→	7
5	6.25	80.5	47.3	34.3	29.7	29.2		31.5			35 ^w
6	3.13	117.1	77.6	43.6	37.8	3.3		31.5			31 ^w
7	1.57	135	55.4	47.7	44	37.7			34.1		25 ^w
8	0.78	203.7		108.8	77	43.8			38.1		19 ^w

† computed as percentage of STANDARDS given in Fig. 14 and Table XII

TABLE XIX Effect of Benzene Extraction During Conversion of Prothrombin to Thrombin in Ceph Ca AcG Activated Eluate

Clotting times (seconds) at 27°C for 0.2 ml (or equivalent) test mixture + 0.2 ml 1% bovine fibrinogen after stated periods subsequent to adding agent cited

TEST SERIES	AGE of MIXTURE	ADDED AGENT	INCUBATION PERIOD (minutes)					
			1	5	10	20	30	45
1		Activ mix		31	28.8	30.3	32.7	
2	(1) 5 m	Benzene extr	30.7*	47.8	79	150	169	172
3	(2) 70 m	Re-act mix		174.5	185	242.4		

TABLE XX Treatment of Thrombin and Fibrinogen with (A) Decalcifying (Ion Exchange) Resin (B) Benzene

Clotting times (seconds) before and at stated periods after the treatment noted 0.2 ml Upjohn's bovine thrombin + 0.2 ml fibrinogen

Treatment	<u>Before</u> treatment	<u>After treatment period of</u>				
		10 min	20 min	30 min	60 min	5 hr
A	13.7	15.7			29	(12.9)*
B	10.9	13.5	14.3	11.2	-	

*Treated thrombin tested on untreated fibrinogen

TABLE XXIV
A Assays of Clotting Factors in (Howell Type) Prothrombin and Precursor Plasmas (Dog)

For methods see text Percentage units refer to standards (Fig 16) obtained with normal (100%) dog oxalated plasma
Recovery is computed (%) from (4) and (1) allowing for two fold dilution in (4)

PREPARATION	FIBR	THR	PROTHROMBIN		PROCONV	PROACCEL	PTC	AHF
			2 stage	1 stage	1 stage	1 stage		
			u/ml					
1 Citr Plasma	+ (9.3)	0	254	100%	96%	80%	++	++
2 Berkefeld Pl	+ (10)	0	100	56%	68%	25%	NT	NT
3 Defibr Plasma	trace	0	144	64%	48%	22%	NT	NT
4 Prothrombin	0	tr	37	10%	20%	25%	++	+
		45						
Recovery			29%	20%	40%	50%	?	?

B Antithrombin Test on Howell Type Prothrombin(PIo)

Clotting times (seconds) for 0.2 ml fibrinogen + 0.2 ml sample of mixture of equal (1 ml) vols
PIo + thrombin (bovine 20 units/ml) tested after stated incubation periods 28°C

INCUBATION PERIOD (minutes)	1/4	5	10	15	20	30
	17.6	18.8	17.9	18	17.3	17.1
Test Clotting Times						

TABLE XXII Effects of Varying Cephalin Concentrations on the Clotting Power Developed in Thrombic (i.e. Prothrombin + Ca) Mixtures

Clotting times (seconds) at 38°C for 1 ml fibrinogen + 0.5 ml T M tested at stated incubation periods (see text)

	FINAL DIL O ⁻ CEPH	INCUBATION PERIOD (minutes)					RETESTED (4 hr) 1:1000 Ceph added
		5	30	60	120	180	
1	1 × 10 ⁻³	20	7	7	7	7	12
2	1 × 10 ⁻⁴	23	10"	9	9	10	12
3	1 × 10 ⁻⁵	90	23	18	15	15	12
4	1 × 10 ⁻⁶	720"	140	50'	30	30	12
5	1 × 10 ⁻⁷	2160	680	330	73'	65	12

TABLE XXIII Effects of Varying Thromboplastin Concentration on Activation of Prothrombin with Special Reference to Thrombin Yield

End point clotting times (seconds) at 25 ± 2°C after stated incubation periods (see text)

	FINAL CONC OF THROMBOPLASTIN	END POINT CLOTING TIME	REQUIRED INCUBATION PERIOD
1	1:500	4	1 hr
2	1:1000	4	2 hrs
3	1:4000	6	18 hrs
4	1:40,000	8	3 days
5	1:400,000	10	4 days

TABLE XXVI Effects of Increasing Amounts of Calcium on Thrombin Formation

Same test conditions as in Table XXV (Expt 23) except for the increased concentrations of CaCl_2

T.M.	Ca CONC	Ca CONC	INCUBATION PERIOD (minutes)					
	IN T.M.	IN T.M. + F	1	5	10	20	30	60
I	8 mM	25mM	39.8	38.2	<u>32</u>	35.3	35.8	36.1
II	10 mM	25 mM	43.3	40.7	<u>40</u>	45.9	41.7	44.5
III	15 mM	25 mM	50.6	<u>37.4</u>	37.4	37.5	40.5	41.1
IV	20 mM	25 mM	55.3	<u>35.9</u>	38.1	39.3	41.6	40.8
V	25 mM	25 mM	81.9	<u>45.4</u>	47.3	48.2	46.1	45.5

TABLE XXVII Effects of Varying Amounts of Ca^{++} During Prothrombin Activation

T.M. 5 ml total vol containing in borate buffer (pH 7.7) 1 ml 0.1% Prothrombin H (ref. [156]) + 0.25 ml 0.5% Difco's (commercial) rabbit brain thromboplastin + 0.25 ml CaCl_2 (final molarity cited)

Clotting times (seconds) at 29°C for 0.25 ml T.M. + 0.5 ml B.F. (0.5%) + 0.25 diluent (containing the same amount of tpin as in T.M. and exactly enough CaCl_2 to bring the final concentration of calcium to 0.0125 M in each (T.M. + B.F.) final clotting test mixture)

T.M.	Ca IN	INCUBATION PERIOD (minutes)						
	T.M.	1/4	5	10	15	30	60	120
1	0	127	126	125	125	129	139	145
2	0.002 M	104	12	4.5	4	4	4	4
3	0.005 M	99	6	4.5	4	4	4	4
4	0.025 M	98	6	5	4.5	4	4	4
5	0.05 M	139	75	52	21	7.5	5	4

TABLE XXV Effects of Decreasing Amounts of Calcium on Activation of Prothrombin (and Proconvertin) Containing Eluate in the Presence of Added AcG and Tissue Thromboplastin

T.M.^{1st} 10 ml (total) volume in imidazole buffered saline (pH 7.3) containing 0.2 ml eluate 0.1 ml AcG†
 (1st) 0.5 ml Soluplastin and CaCl₂ to concentration (milli Molar) stated
 Clotting tests: 0.4 ml fibrinogen + 0.2 ml (adjusted) Ca⁺⁺ + 0.2 ml T.M. at stated incubation periods
 Clotting times (seconds) at 26°C

T. M. added/10 ml T. M.	ml 0.02 M Ca	Conc Ca in T. M. (mM)	Conc Ca* in T. M.+F. (mM)	INCUBATION PERIOD (minutes)						
				1	5	10	15	20	30	60
1	4 (ml)	8 (mM)	5 (mM)	47.9	43.7	<u>42.3</u>		44.2'	47.9	53.7
2	2 (ml)	4 (mM)	5 (mM)	58.6'	44.7	<u>42.6</u>		42.9	45.9	43.3'
3	1 (ml)	2 (mM)	5 (mM)	75.8	64	<u>42.2</u>	-		43.4	46.2'
4	0.5 (ml)	1 (mM)	5 (mM)	105.1	46.1	44.5	<u>42.4</u>		46.8'	46.5
5	0.25 (ml)	0.5 (mM)	5 (mM)	185.7	89.8	57.4	50.2	47.7	<u>42.7</u>	46.6
6	0 (buff only)	0	5 (mM)	311'	254	272		256"	319	280'

TABLE XXIX Stability of Purified Prothrombin Solution Activation by (1) Ca Alone (2) Ca + Thromboplastin (see text)

Clotting times (seconds) at 25°C of 0.5 ml fibrinogen + 0.25 T M after stated incubation periods

T M	DATE OF EXPERIMENT	ACTIVATOR ADDED	INCUBATION PERIOD							
			1 m	10 m	30 m	1 hr	2 hr	6 hr	1 d	7 d
1	Oct 8 1946	Ca (only)	112	87	66	48	38	16	9	3
2	Oct 8 1946	Ca+tpln A	68	14	6	5	3	3	3	3
3	Dec 9 1946	Ca+tpln D	20	12	8	4	3	3	3	3

TABLE XXVIII Spontaneously Formed Thrombin (II)
Compared with Rapidly (Ca+pln) Activated
Prothrombin (I)

Clotting times (seconds) at 25°C for serial dilutions
of the two thrombins on the same fibrinogen under
the same test conditions

MIXTURE	RELATIVE STRENGTHS (by dilution)					
	100%	50%	25%	10%	5%	1%
I	4	8	11	22	40	84
II	4	8	11	22	42	102

TABLE XXXII Thromboplastic and Anti Thromboplastic Assays on Various Lipoids and their Contaminants

Synthetic phosphatides are prepared as sodium salts

Testing methods are described in the text

Results are indicated by the following symbols

+ +++ = varying degrees of thromboplastic (TPL IC) activity

± = slightly enhances action of standard cephalin

- = varying degrees of inhibition (ANTI TP) of cephalin

N T = not tested

No.	DESCRIPTION OF PREPARATION	TPL IC	ANTI TP
1	Standard Cephalin (from dog brain) a crude mixture of phosphatides etc	+++	(>50γ/ml)
2	1 2 distearoyl α cephalin synthetic >95% pure	0	0
3	1 2 - dipalmitoyl α cephalin synthetic >95% pure	0	0
4	1 3 dimyristoyl β- cephalin synthetic >95% pure	0	0
5	stearal acetal phosphatidyl ethanolamine synthetic >95% pure	0	0
6	myristal acetal phosphatidyl ethanol amine synthetic 80-95% pure		
7	myristal acetal phosphatidyl ethanol amine repurified from 6 >99% pure	N T	0
8	palmital acetal phosphatidyl ethanol amine synthetic >99% pure (group analyses)	±	0
9	trace impurity (A ₁) from preparation 6	N T	
10	trace impurity (A ₂) from preparation 6	N T	
11	trace impurity (B ₁) from preparation 6	N T	
12	trace impurity (B ₂) from preparation 6	N T	0
13	mixture of 7 + 9	N T	
14	mixture of 7 + 11	N T	0
15	mixture of 7 + 9 + 11	N T	
16	mixture of 9 + 11	N T	
17	trace impurity (I) from preparation 8	0	-
18	trace impurity (II) from preparation 8	±	0

TABLE XXX Stability of Prothrombin and the Thrombin Formed from it by Simple Recalcification

For Details see text Clotting times (seconds) of 0.5 ml fibrinogen + 0.25 T.M. (dil) at stated periods

Series (A) T.M. (2 ml) diluted with 0.25 ml buffer before test

Series (B) T.M. (2 ml) maximally activated with 0.25 ml tissue thromboplastin (within 1 hr) before testing with B.F. End point clotting times cited $23 \pm 2^\circ\text{C}$

TEST SERIES	AGE OF T. M. BEFORE TESTING				
	1 hr.	1 day	2 days	3 days	8 days
A	250	85	43	25'	4
B	4	4	4	4	4

TABLE XXXI Thrombin Formation in Presence of Various Thromboplastic Agents from Brain Tissue

Thrombic mixtures (T) = 4 cc prothrombin + 0.5 cc borate buffer + 0.25 cc N/10 CaCl_2 + 0.25 cc cited agents incubated for periods indicated Clotting times (seconds) * at 25°C pH - 7.75 for 0.5 cc T + 1.0 cc prothrombin free fibrinogen The P lipids are 1:1000 suspensions in borate buffer

T	THROMBOPLASTIC AGENT	INCUBATION PERIOD (minutes)						
		10'	20'	30'	60'	90'	120'	150'
0	(Calcium only)	±	±	±	±	±	±	±
1	Brain Susp	45	28	27	27	27	28	30
2	P lipid V	+	320	180	63	53	48	49
3	P lipid III	+	760	340	108	78	76	78
4	P lipid I	+	570	380	140	90	82	82
5	P lipid IV	245	120	70	38	39	40	42

* + clot starting in $\frac{1}{2}$ 1 hr and becoming solid later

+ weak clot starting in several hours and incomplete in 24 hours

TABLE XXXIV Effects of Cofactor on Action of Heparin During and After Thrombin Formation

Details of experiments are given in the text
Clotting times (seconds) at 38°C for appropriately diluted fibrinogen (see text) after incubation of T.M.'s for the stated periods at 7.5°C

T.M.	INHIBITOR	INCUBATION PERIOD (minutes)					TESTS ON		No
		5	90	120	T.M. (I) at 1 hr.	T.M. (II) at 1 hr.			
1		95	22	18	18	18			5
2	Heparin		+	900	30	23		19	6
3	Cofactor	40	20	19	20	25		28	7
4	Hep + Cof	∞	∞	∞	∞	∞		23	8

TABLE XXXV Inhibitory Effects of Heparin During Prothrombin Activation by Tissue Thromboplastin

See text for details of experiment
Clotting times (seconds) at 25 ± 2°C for incubated mixtures and the fibrinogens noted in the text

T.M. AND TEST	INCUBATION PERIOD							
	5 min.	15 min.	30 min.	1 hr.	2 hr.	8 hr.	1 d.	3 d.
1a (without heparin)	63	42	20	9	6	4	4	4
1b (heparin in fibrinogen)	840	60	29	12	8	5	4½	4½
2 (with heparin)	2 hr	375	60	19	13½	10	9	7½

TABLE XXXIII Effects of Heparin on Formation of Thrombin from Recalcified Prothrombin in Presence of Various Thromboplastic Agents

T M (see text) incubated at 15°C for periods stated Clotting times (seconds) at 38°C for 1 ml fibrinogen + 0.5 ml T M Amounts of additives per 5 ml T M

T M	THROMBOPLASTIC AGENT	INHIBITOR	INCUBATION PERIOD (minutes)					
			1	5	10	20	30	60
1	Cephalin (0.1 mg)			50	20	11	9	9
2		Heparin (0.5 mg)		∞	∞	∞	∞	∞
3	Brain P lipid (0.025 mg)		50	20	10 ¹	8	6	6
4	'	Heparin (0.5 mg)	∞	∞	∞	∞	1800+	360+
5	Tpln Q (0.025 mg lipids)	-	35	16	9	7	7	7
6		Heparin (0.5 mg)	720	16	10	7	7	7
7	Trypsin (0.125 mg)	-	-	30	15	12	11	8
8	"	Heparin (0.5 mg)		97	32	13	11	9

TABLE XXXVII PTC and AHF Assay

- Agents 0 Saline (substrate control)
 I Dialyzed eluate (p 71)
 II Howell type Prothrombin (p 90)
 III Purified AHG (Antihemophilic globulin (p 77))
 IV Normal human plasma (factor control)

Methods are described in the text

The substrates (SUBS) are plasmas from cases deficient in PTC or AHF respectively

AGENT	SUBS	ORIGINAL MIXTURES			AGE OF SERUM	SERUM TESTS		PROT CONS
		(a) additive	(b) c t	(c) " pro		(a) c t	(b) " pro	
0	PTC	saline (control)	21 3	126	½ hr	23 9	86	32"
0	AHF		22	115	1 hr	22 7	103	10"
I	PTC	eluate (1 l)	22 3	112	½ hr	27 8	46	59%
I	AHF		20 9	131	1 hr	31 3	35	73%
II	PTC	Howell Pro (1:1)	20 5	137	½ hr	29 7	40	71%
II	AHF		21 3	126	1 hr	26 5	54	57%
III	PTC	AHG (1")	22 4	109	½ hr	51 7	8	96%
III	AHF	AHG (0.001")	21 1	129	1 hr	36 9	22	83"
IV	PTC	Plasma (1 5)	21 9	117	½ hr	25 5	65	44½%
IV	AHF	Plasma (1 10)	22 7	105	1 hr	63	3	97"

TABLE XXXVI Effects of Trypsin on Activation of Prothrombin in Presence of Various Added Activators
Experimental details given in text
Percentages of prothrombin activated after stated incubation periods 25°C
T M 's: 5 ml total vol containing 4 ml Pro D (0.2%) + stated additives

T M	ACTIVATORS ADDED (ml)			INCUBATION PERIOD								
	CaCl ₂ 0.1 M	trypsin 0.1%	trypsin 40 u/ml	5 min	15 min	30 min	1 hr	3 hr	6 hr	24 hr	30 hr	48 hr
I	0.25				±	±	+	1%	4%	40%	80%	100%
*II		0.5	-		+	1%	1%	8%	20%	2%	+	
*III	0.25		0.5	6%	25%	35%	60%	100%	80%	4%	1%	
IV	0.25	0.75		40%	80%	100%	100%	100%				
V	0.25	0.25		1%	5%	9%	20%	30%	35%	70%	80%	100%
*VI	0.25	0.25	0.5	35%	42%	60%	75%	100%	50%	3%	1%	

*NOTE. Clot lysis occurred in 3 days in tubes containing trypsin

TABLE XL Effects of (A) Weak Thrombin and (B) Fibrinolysin upon the Thromboplastic Action of Platelets

Experimental details given in text

T.M.: 0.2 ml Pro 0.1" + 0.2 ml AcG 0.1" + 0.5 ml CaCl_2 (0.05 M) the calcium being added after the other activators cited making with the borate buffer (pH 7.7) diluent a total 5 ml vol

Clotting times (seconds) at 26°C for 0.5 B F (1") + 0.25 T M at the stated incubation periods

T M	ACTIVATORS (ml/5 ml T.M.)	INCUBATION PERIOD (minutes)				
		5	10	20	40	60
0	0 (control)	885	913	920	970	1010
1	Platelets (0.2)	810	620	330	190	97
2 (A)	Plat (0.2) + Thr* (0.5)	145	52	22	12	9
3 (B)	Plat (0.2) + Lysin (0.6)	60	46	15	12	9

* Special thrombin see text

TABLE XXXVIII Potentiation of Thromboplastic Action of Platelets by (A) Antihemophilic Globulin and (B) Trypsin

Experimental details given in text All T M 's contain 0.1 ml dialyzed eluate 0.1 ml AcG (1:5) and 0.5 ml 0.02M CaCl_2 with imidazole buffered saline to 5 ml total volume

Clotting times (seconds) at 28°C for 0.2 ml fibrinogen + 0.2 ml T M after stated incubation periods

T M	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes)					
		1	5	10	20	30	60
(A) 1	(0.2) platelets	2 hr	495.2	65	31.9"	<u>30.3</u>	32.3
2	(0.2) platelets (0.2) AHG (1%)	2 hr	92.6	44.9	42.7	<u>40.1</u>	45
3	(0.2) AHG (1%)	1 hr	582.5	349.6	<u>164.2</u>	246.1	
(B) 4	(0.2) platelets (0.1) trypsin	155.5	42.1	<u>35.7'</u>	39.8	37.5	38.8
5	(0.1) trypsin	-	2 hr	1609+	791	570	445
6	(0.1) trypsin (0.2) AHG (1%)	732.6	242.2	195.4	<u>172.6</u>	174.2	

TABLE XXXIX Potentiation of Thromboplastic Action of Platelets by (C) Weak Thrombin and (D) Fibrinolysin

Experimental details given in text All T M 's contain 0.1 ml dialyzed eluate 0.1 ml AcG and 0.5 ml 0.02M CaCl_2 with imidazole buffered saline to 5 ml total volume

Clotting times (seconds) at 26°C for 0.2 ml fibrinogen + 0.2 ml T M after stated incubation periods

T M	(ml) OTHER ADDITIVES	INCUBATION PERIOD (minutes)					
		5	10	20	30	60	90
(C) 7	(0.2) platelets (0.2) thrombin	191	53.5	29.5	30.3	26.9	27.7
(D) 8	(0.2) platelets (0.1) lysin	194	66.6	39	34.4	31.3	29.5
on (control)		2 hr	2 hr	2 hr	1560	820	

TABLE XLI Effects of Anti Proteases on Thromboplastic Action of Platelet Trypsin Additions to Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7.7) containing 0.2 ml Pro (0.1%) + 0.2 ml AcG (0.1%) + 0.1 ml CaCl₂ (0.05M) added after the various other activators and inhibitors cited. The asterisk (*) with the inhibitor denotes a 15 minute (room temperature) pre incubation with the activator system

Clotting times (seconds) at 25°C for 0.5 ml B.F. (1%) + 0.25 ml T.M. at successive incubation periods Clot-lysis followed over 3 weeks

T M	ACTIVATORS	INHIBITORS	INCUBATION PERIOD (minutes)					CLOT LYSIS
			5	10	20	40	60	
1	platelets	0	720	540	270	100	65	0
2	trypsin	0	184	140	95	58	51	3 d
3	platelets + trypsin	0	47	29.5	26.5	23.5	23.2	0
4	platelets + trypsin	P I	660	240	175	113	96	0
5	platelets + trypsin	Alin	- 0	470	205	103	71	0

TABLE XLVI Lack of Effects of Anti Proteases on the Thrombin Fibrinogen Reaction

Clotting times (seconds) at 25°C for 0.5 ml fibrinogen + 0.25 ml thrombin (5 units/ml) + 0.25 anti protease (or buffer in control) the last two* being pre incubated with thrombin for the periods noted

	ADDITIVE	PRE INCUBATION PERIOD (minutes)				
		15	10	20	30	60
0	Buffer	14.8	15	15	14.6	15.3
1	P I	17	18.5	18.5	19.5	20.5
2	S B I	14.2	16	17	17.1	17
3	N B I *	15.2	16	16.5	16.7	16.8
4	Afln *	15	16	16	15.5	16.4

TABLE XLVII Proteolytic Activities of Old Enzyme Preparations and Their Inhibition by Anti Proteases

Experimental details and descriptions of the enzyme preparations are given in the text

The pancreatic inhibitor (P I) acts immediately whereas the navybean inhibitor (N B I *) and antifibrinolysin (Afln *) require 15-20 minute pre incubation with the enzyme before adding the fibrinogen to make the L.M

Clotting times (seconds) at 28°C for 0.2 ml thrombin (20 units/ml) + 0.2 ml L.M after incubation periods stated

L M	ADDITIVES	INCUBATION PERIOD (minutes)		
		10	20	30
(A) 1	0 (buffer)	14.2	14	14.3
2	trypsin	540	840	∞
3	trypsin + N B I *	14	15	14
4	trypsin + P I	15.7	22	28
(B) 5	fibrinolysin	∞	∞	∞
6	lysin + N B I *	21	22	18
7	lysin + Afln *	13	13.8	14.8

TABLE XLIV Effects of Anti-Proteases on the Action of Tissue Thromboplastin in Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7.7) contain 0.2 ml Pro (0.1%) + 0.5 ml tpin A + 0.2 ml AcG (0.1%) + 0.5 ml CaCl₂ (0.05M) added after the various inhibitors cited. In (4) the thromboplastin was pre incubated for 15 minutes at 25°C with the anti-fibrinolysin *

Clotting times (seconds) at 25°C for 0.5 ml B.F. (1%) + 0.25 ml T.M. at successive incubation periods

T M	INHIBITOR ml (conc.)	INCUBATION PERIOD (minutes)				
		5	10	20	40	60
1	0	22.5	17.1	15.2	15	14.2
2	S B I 0.2 (0.01%)	450	420"	450'	450	380
3	P I 0.2 (0.1%)	28.8	23.3	21.8	21.5'	20
4	Afln * 0.3 (0.5%)	28.5	22.4	20.6'	21.2	21"

TABLE XLV Effects of Anti Proteases on the Action of Cephalin in Thrombin Forming Systems

T.M.'s 5 ml in borate buffer (pH 7.7) contain 0.2 ml eluate (dialyzed) + 0.1 ml cephalin (0.1%) + 0.2 ml AcG (1:8) + 0.5 ml CaCl₂ (0.04M) added after inhibitors stated. In test (4) the cephalin was pre incubated for 15 minutes at 28°C with the N B I *

Clotting-times (seconds) at 28°C for 0.2 ml (dog) fibrinogen + 0.2 ml T.M. at successive incubation periods

T M	INHIBITORS ml (conc. %)	INCUBATION PERIOD (minutes)			
		5	10	20	30
1	0	56.5	52	50.8	56.4
2	S B I 1.0 (0.001)	222	156.5	149.1	163
3	P I 0.1 (0.1)	48.8	50.3	57	59.8
4	T B I 0.1 (0.1)	46.2	44.2	49.4'	53.6

TABLE XLIX Effects of Heparin on Thrombin Forming Mixtures Activated by (I) Platelets; (II) Platelets + Trypsin; or (III) Tissue Thromboplastin (See text)

T.M.I. 5 ml in imidazole buffered saline (pH 7.3) containing 0.2 ml eluate (dialyzed) + 0.1 ml AcG (1:8) + 0.1 ml (I) platelets OR (II) platelets + 0.5 ml trypsin (0.02%) OR (III) Soluplastin (0.2 ml) + 0.5 ml (a) buffered saline OR (b) heparin (2 units/ml) + 0.5 ml CaCl_2 (0.04M)

Clotting times (seconds) at 28°C for 0.1 ml fibrinogen + 0.1 ml (c) heparin (0.2 units/ml) OR (d) buffered saline + 0.1 ml T.M. after stated incubation periods

T.M.	ACTIVATORS	HEPARIN in T.M.	INCUBATION PERIOD (minutes)										
			3	5	8	10	15	20	25	30	35		
I 1 (a)	(d) Platelets	0		64		29.9		29.2		29.5			
I 1 (a)	(c) Platelets	0	98.6		24.1				23.6				24
I 2 (b)	(d) Platelets	+								1000+			
II 3 (a)	(c) Plat + tryp	0	61.4	40.6		36.4						35.8	34
II 4 (b)	(d) Plat + tryp	+	172.5	64.5		47.6		35.4				37.2	
II 5 (a)	(d) Trypsin	0	570	495		392	352	407		420			
III 6 (b)	(d) Soluplastin	+	37.1	35.8		31.7						30.1	

TABLE XLVIII Effect of Washed Platelets as an Accelerator of the Activation of AcG Poor Prothrombin

Reagents and experimental details are described in the text

T.M. 5 ml in borate buffer (pH 7.75) containing 0.2 Pro (0.1%) + 0.5 ml CaCl_2 (0.05M) + the various activators noted

Clotting times (seconds) at 26°C of 0.5 ml B.F. (1%) + 0.25 ml T.M. at successive incubation periods

T.M.	ACTIVATORS ml (dilution)	INCUBATION PERIOD (minutes)				
		5	10	20	40	60
1	Tpin B 0.5	420	300	202	141	110
2	Tpin 0.5 + Plat 0.2	390	240	80	22.4	15
3	Tpin 0.5 + AcG 0.1 (0.001%)	300	140	52	25.8	20.3
4	Tpin 0.5 + AcG 0.1 (0.1%)	11.4	9.4	8.0	7.8	7.6

TABLE XLIV Effects of Heparin on Thrombin Forming Mixtures Activated by (I) Platelets; (II) Platelets + Trypsin or (III) Tissue Thromboplastin (See text)

Tube: 5 ml in imidazole buffered saline (pH 7.3) containing 0.2 ml eluate (dialyzed) + 0.1 ml AcG (1:8) + 0.2 ml (I) platelets or (II) platelets + 0.5 ml trypsin (0.02%) or (III) Soluplastin (0.2 ml) + 0.5 ml (a) buffered saline or (b) heparin (2 units/ml) + 0.5 ml CaCl_2 (0.04M)

Clotting time (seconds) at 28°C for 0.2 ml fibrinogen + 0.1 ml (c) heparin (0.2 units/ml) or (d) buffered saline + 0.1 ml T.M. after stated incubation periods

T.M.	ACTIVATORS	HEPARIN In T.M.	INCUBATION PERIOD (minutes)									
			1	5	8	10	15	20	25	30	35	
I 1 (a)	(d) Platelets	0		64		29.9		29.2		29.5		-
I 1 (a)	(c) Platelets	0	98.6		24.1				23.6			24
2 (b)	(d) Platelets	+						-		1000+		-
II 3 (a)	(c) Plat + tryp	0	61.4	40.6								34
4 (b)	(d) Plat + tryp	+	172.5	64.5		36.4						
5 (a)	(d) Trypsin	0	570	495		47.6		35.4				
						392	352	407				420
III 6 (b)	(d) Soluplastin	+	37.1	34.8		31.7						30.1

TABLE L Effects of Platelets on the Thrombin Fibrinogen Reaction

Method: 0.2 ml thrombin (conc. cited) + (1) 0.1 ml platelets or
(2) 0.1 ml saline + 0.2 ml fibrinogen

Clotting-times (seconds) at 26°C

ADDED THR (units/ml):	20	10	5	2.5	1.25
(1) with platelets	10.5"	17.1"	31.5"	59.0'	129 (130)
(2) control with saline	10.5	17.8	31.0	59.5'	105" (105.8)

TABLE LI Platelet Utilization During Clotting of (A) Normal and (B) Hemophilic Blood

Platelet counts ($\times 1000/\text{mm}^3$) after stated times in siliconed tubes at 37°C (I) in clotting bloods (II) in sequestrene plasma ('aging' control)

TIME (minutes)	(A) NORMAL BLOOD (I)	(B) HEMOPHILIC BLOOD (I)	(II) CONTROL
0	320	182	
1	290	154	
2	265	192	
3	196	132	
4	238	150	
5	103	125	
6	106		
	(clotting)		
7	34	137	
8	31	-	
9	19	-	
10	31	143	
12.5	15	136	
15	18	160	
		(clotting)	
30	3.3	51	
45	4.2	14	
60	3.7	2.4	(A) 252
75		2	
90	-	1.7	
120		4	(B) 174

TABLE LH GROUP I (A) Normal adults; (B) Healthy parturient mothers; (C) Normal newborn infants (cord bloods)

1 No (Sub group) 2 Cases	>50 (A) Normal Adults	32 (B) Mothers	32 (C) Newborns
3 Age	19 55	15 43	0
Sex	F M	F	F M
Race	W C	23W 9C	23W 9C
4 Date(s) Exam	1950 55	1953 55	1953 55
5 Duration Bleeding	neg	neg	neg
6 Fam Hist of Bleeding	neg	neg	neg
7 Bleed Tendency	neg	neg	neg
8 Tourniquet Test	neg	N T	N T
9 Bleed time (min)	6 (2 10)		
Clotting Time:			
10 glass (min)	9 (5 13)	7 (2, 12)	5 (1 10)
11 silicone (min)	33 (15 50)	24 (6 60)	12 (3 33)
12 Clot Retraction	+++(+)	+++(+)	++++ +
13 Fibrinolysis	neg	neg	neg (+ 2 cases)
14 Platelet Count x 1000/mm ³	249 (143 356)	241 (105 446)	271 (136 523)
15 Platelet Tpin *	100 (65 150)	80 100+	80 100+
16 Platelet Accel *	100 (40 150)	60 100+	50 100+
			(one 10)
17 Serum Serotonin*	100 (50 150)	N T	N T
18 Platelet Agglut	neg	N T	N T
19 Plasma Thr C T	15 (13 17)	16.5 (av)	25.5 (av)
20 Serum Antithrombin	variable	normal	normal
21 Fibr mg/100 ml	364 (200 540)	652 (340 1020)	344 (192 800)
22 AHF*	100 (60 135)	60 100+	60 100+
23 PTC*	100 (60 135)	110 (83 143)	59 (15 90)
24 Inhibitors etc	neg	neg	neg
25 Prothr Cons %	95 (90 99)	93 (80 96)	83 (55 95)
26 Prothr Time*	100 (74 133)	119 (68 133)	119 (56 133)
27 Prothrombin*	100 (70 136)	115 (81 150)	43 (30 59)
28 Proconvertin*	100 (64 138)	135 (100 216)	51 (22 80)
29 Proaccelerin*	100 (60 150)	70 100+	100 300
30 Remarks	Med School personnel	Parity 1 11	125 others excluded

* percent of standard normal used throughout Tables LH LVIII

TABLE LIII A Group II Congenital (Familial) Thrombocytopenias

1 2	No CASE	Case 1 A B		Case 2 D D
3	Age	2	3	9
	Sex	M	M	M
	Race	C	C	C
4	Date(s) Exam	1 Feb '54	20 Apr '55	20 Apr '55
5	Duration Bleeding	1 16 mo	-	-
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	0	0
8	Tourniquet Test	+++	neg	+(+)
9	Bleeding time (min)	60	2½	6½
	Clotting Time			
10	glass (min)	6½	6½	5 3/4
11	silicone (min)	6½	7½	29½
12	Clot Retraction	0 ±	+++	++
13	Fibrinolysis	0	0	0
14	Platelet Count x 1000/mm ³	38	320	98
15	Platelet Tpin *	N T	50	20
16	Platelet Accel *	N T	80	10
17	Serum Serotonin*	N T	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	±	±	±
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	320	N T	520
22	AHF*	100	100	100
23	PTC*	80	80	70
24	Inhibitors etc	N T	N T	N T
25	Prothrombin Cons %	95	100	95
26	Prothrombin Time*	125	93	88
27	Prothrombin*	111	106	93
28	Proconvertin	95	100	110
29	Proaccelerin*	100+	100+	100
30	Remarks	Before treatment	After splenect	

* percent of standard normal

TABLE LIII B Group III Hereditary Thrombasthenia (3) and Pseudo hemophilia (4 5)

1 No	Case 3	Case 4	Case 5
2 CASE	(N P)	(A C)	(S C)
3 Age	16	52	20
Sex	F	F	F
Race	W	W	W
4 Date(s) Exam	9 June '53	23 Apr '55	23 Apr '55
5 Duration Bleeding	birth	33 yrs	?
6 Fam Hist of Bleeding	+	+	+
7 Bleeding Tendency	+++	+(+)	+(+)
8 Tourniquet Test	++++	+	++
9 Bleeding time (min)	60	1	6½
Clotting Time			
10 glass (min)	9	6 3/4	9½
11 silicone (min)	22	31	N T
12 Clot Retraction	0	+++	+++
13 Fibrinolysis	neg	neg	neg
14 Platelet Count			
x 1000/mm ³	230	238	104
15 Platelet Tpln *	100	50	25
16 Platelet Accel *	N T	50	20
17 Serum Serotonin*	100	N T	N T
18 Platelet Agglut	N T	N T	N T
19 Plasma Thr C T	norm	N T	N T
20 Serum Antithrombin	+	N T	N T
21 Fibr mg/100 ml	520	720	700
22 AHF*	100	100	100
23 PTC*	85	90	60
24 Inhibitors etc	neg	neg	neg
25 Prothrombin cons "	95	>95	>95
26 Prothrombin Time*	94	85	71
27 Prothrombin*	89	90	100
28 Proconvertin*	111	N T	100
29 Proaccelerin*	62	N T	90
30 Remarks	Glanzmann's Thrombasthenia	Pseudohemophilia	

* Percent of standard normal

TABLE LIII A Group II Congenital (Familial) Thrombocytopenias

1	No	Case 1		Case 2
2	CASE	A	B	D D
3	Age	2	3	9
	Sex	M	M	M
	Race	C	C	C
4	Date(s) Exam	1 Feb '54	20 Apr '55	20 Apr '55
5	Duration Bleeding	1 16 mo	-	-
6	Fam Hist of Bleeding	+	+	+
7	Bleeding Tendency	+++	0	0
8	Tourniquet Test	++++	neg	+(+)
9	Bleeding time (min)	60	2½	6½
	Clotting Time			
10	glass (min)	6½	6½	5 3/4
11	silicone (min)	6½	7½	29½
12	Clot Retraction	0 ±	+++	++
13	Fibrinolysis	0	0	0
14	Platelet Count x 1000/mm ³	38	320	98
15	Platelet Tpln *	N T	50	20
16	Platelet Accel *	N T	80	10
17	Serum Serotonin*	N T	N T	N T
18	Platelet Agglut	N T	N T	N T
19	Plasma Thr C T	±	±	±
20	Serum Antithrombin	+	N T	N T
21	Fibr mg/100 ml	320	N T	520
22	AHF*	100	100	100
23	PTC*	80	80	70
24	Inhibitors etc	N T	N T	N T
25	Prothrombin Cons *	95	100	95
26	Prothrombin Time*	125	93	88
27	Prothrombin*	111	106	93
28	Proconvertin	95	100	110
29	Proaccelerin*	100+	100+	100
30	Remarks	Before treatment	After splenect	-

* percent of standard normal

TABLE LIII B Group III Hereditary Thrombasthenia (3) and Pseudo hemophilia (4-5)

1 No	Case 3	Case 4	Case 5
2 CASE	(N P)	(A C)	(S C)
3 Age	16	52	20
Sex	F	F	F
Race	W	W	W
4 Date(s) Exam	9 June '53	23 Apr '55	23 Apr '55
5 Duration Bleeding	birth	33 yrs	?
6 Fam Hist of Bleeding	+	+	+
7 Bleeding Tendency	+++	+(+)	+(+)
8 Tourniquet Test	++++	+	++
9 Bleeding time (min)	60	1	6½
Clotting Time			
10 glass (min)	9	6 3/4	9½
11 silicone (min)	22	31	N T
12 Clot Retraction	0	+++	+++
13 Fibrinolysis	neg	neg	neg
14 Platelet Count			
x 1000/mm ³	230	238	104
15 Platelet Tpln *	100	50	25
16 Platelet Accel *	N T	50	20
17 Serum Serotonin*	100	N T	N T
18 Platelet Agglut	N T	N T	N T
19 Plasma Thr C T	norm	N T	N T
20 Serum Antithrombin	+	N T	N T
21 Fibr mg/100 ml	520	720	700
22 AHF*	100	100	100
23 PTC*	85	90	60
24 Inhibitors etc	neg	neg	neg
25 Prothrombin cons *	95	>95	>95
26 Prothrombin Time*	94	85	71
27 Prothrombin*	89	90	100
28 Proconvertin*	111	N T	100
29 Proaccelerin*	62	N T	90
30 Remarks	Glanzmann's Thrombasthenia	Pseudohemophilia	

* percent of standard normal

TABLE LIV Group IV Summary of Data on 25 Thrombocytopenics
(Acquired)

		FINDINGS				REMARKS
		Pos.	Neg.	?	N.T.	
1	Nos 6 30					Diag y text
3	Ages 17 mo 78 yr					
	Sex 11 F 14 M					
	Race 22 W 3 C					
5	Duration Bleed 1 d 8 yr	19	1		5	
7	Bleeding Tend +++(+)	10				
	++	7				
	+	2				
	0	-	1	1	4	
8	Tourn Test ++++ +	19	3	2†	1	†Negroes
9	Bleed Time >10 min	17	7	1†		†9 min
	Clotting Time					
10	glass >13 min	1	21	3†		†12½ 10½'
11	silicone >50 min	10	10	3†	2	†48 47'
12	Clot Retr (glass) ++ 0	17	3		5	
14	Platelet Count					
	x 1000/mm ³ <100 000	24		1†		†174 000
15	Platelet Tpln* <10	18	1	1†	5	†30
16	Platelet Accel* <10	19			6	
17	Serum Serotonin* <50	9	3		13	
19	Plasma Thr C T >2x norm	15		8†	2	†slight
21	Fibr (a) <200 (decr)	0	14		2	
	(b) >540 (incr)	9				
22	AHF* <60%		22	1†	2	†25 in ♀
23	PTC* <60%		21	2†	2	†60 65
25	Prothrombin Cons <90%	21	3		1	
26	Prothrombin Time* <65		24	1†		†61
27	Prothrombin* <65	3†	21		1	†52 62
28	Proconvertin* <65	1†	23		1	†55
29	Proaccelerin* <60	3†	22			†30 50

*percent of standard normal

TABLE LV Group V: Thrombocytosis

1 No	31	32	33
2 Cases	R G	J L H	E M
3 Age	63	64	23
Sex	M	M	M
Race	C	W	W
4 Date(s) Exam	23 June '55 (27 Aug '56)	30 Mar 54 (8 Apr '55)	15 Dec 54
5 Duration Bleeding	2 weeks	?	?
6 Fam Hls of Bleed	neg	neg	neg
7 Bleeding Tend	++	++	0
8 Tourn Test	neg	neg	neg
9 Bleeding Time (min)	1½ 4	3½	2
Clotting Time			
10 glass (min)	11½	7½	4½
11 silicone (min)	30	48	46
12 Clot Retraction	++++	++++	++++
13 Fibrinolysis	neg	neg	neg
14 Platelet count x 1000/mm ³	2000	1886 (550)	850
15 Platelet Tpln *	100	40	100
16 Platelet Accel *	90 (20)	50	100
17 Serum Serotonin* N T		5	N T
19 Plasma Thr C T	normal	normal	normal
20 Serum Antithr	normal	normal	normal
21 Fibr mg/100 ml	270	510	660
22 AHF*	100	100	100
23 PTC*	90	100	80
25 Prothr Cons %	90	99	90
26 Prothr Time*	85	44 (68)	52
27 Prothrombin	88	68 (48)	60
28 Proconvertin*	100	60 (44)	78
29 Proaccelerin*	60	50 (40)	90
30 Remarks	Carcinoma of stomach general metastases bone marrow hyper plasia incr megakaryocytes	Polycythemia vera treated with p ³² gout	Chronic granulo cytic (Myeloid) leukemia

* percent of standard normal

TABLE LVI A Group VI (A): Leukemics with Thrombocytopenia (22 out of 39 cases studied)

1	Nos	34	55	FINDINGS				Remarks
				Pos	Neg	?	N T	
3	Ages: 13 83 yr Sex: 7 F; 15 M Race 19 W; 3 C							
5	Duration Bleeding: 1 wk 3 mo							
7	Bleeding Tend	++++	2					
		+++	0					
		++	5					
		+	5					
		0		6			4	
8	Tourn Test	++++	0	5	16		1	
9	Bleed Time: >10 min		7	15				
	Clotting Time:							
10	glass: >13 min			20	2	†		†12' (2 cases)
11	silicone: >50 min		15	6	1	†		†45'
12	Clot Retr: ++		16	2	4	†		†clots lysed (see 13)
13	Fibrinolysis		4	18	-			
14	Platelet Count							
	<100 000 x 1000/mm ³		22					
15	Platelet Tpln *: <60		21		-			
16	Platelet Accel *: <40		19	1	1	†	1	†50
17	Serum Serotonin* <50		5	1	-		16	
18	Platelet Agglut		5	6			11	
19	Plasma Thr C T : >2x norm		6	6	10	†		†1.5 2X norm
20	Serum Antithrombin incr		6	13	3	†	-	†'serum' clot
21	Fibr (a) <200 mg		1	12	1	†	1	†230 mg/100 ml
	(b) >540 mg		7	†			-	†560 1180 mg
22	AHF* <60			2	-		20	
23	PTC* <60		1	19	1	†	1	†60
25	Prothrombin Cons <90%		16	5	1	†	-	†90%
26	Prothrombin Time* <65		7	13	2	†	-	†64; 65
27	Prothrombin* <70		2	20	-		-	
28	Proconvertin* <65		3	18	1	†		†66
29	Proaccelerin* <60		12	9	1	†		†60

* per cent of standard normal

TABLE LVI B Group VI (B): Leukemics with Abnormal Platelet Function Tests but no Thrombocytopenia (12 out of 39 cases studied)

1 2	Nos 56 67 Cases	FINDINGS				Remarks
		Pos	Neg	†	N T	
3	Ages: 6 58 yrs Sex: 5F; 7M Race: 11W; 1C					
5	Duration Bleed: (?)				12	
7	Bleeding Tend: ++ +	5	6		1	
8	Tourn Test: ++ +	3	9			
9	Bleed Time: >10 min	1	11			
	Clotting Time:					
10	glass >13 min		10	2†		†12 min
11	silicone: >50 min	9	2		1	
12	Clot Retr ++ 0	3	9			
14	Platelet Count					†118 000
	x 1000/mm ³ : <100 000		12†			445 000/min ³
15	Platelet Tpln*: <60	7	4	1†		†70
16	Platelet Accel*: <40	9	1	2†		†50
17	Serum Serotonin*: <50		-	2†	10	†44 50
18	Platelet Agglut:		2		10	
19	Plasma Thr C T: >2x norm	2	9	1†		†nearly x 2 norm
20	Serum Antithrombin incr	2	10			
21	Fibr: (a) <200 mg (b) >540 mg	0 3†	9			†580 1120 mg/ 100 ml
22	AHF*: <60		2		10	
23	PTC*: <60	1†	11			†45
25	Prothrombin Cons: <90%	4	6	2†		†89 90
26	Prothrombin Time*: <75	4	6	1†	1	†74
27	Prothrombin*: <70	2	10			
28	Proconvertin*: <65	1	11			
29	Proaccelerin*: <60	3	9			

* percent of standard normal

TABLE LVII A Group VII (A) Platelet Problems in Uremics (10 out of 11 cases studied)

1 Nos 68 77	2 Cases	FINDINGS				REMARKS
		Pos	Neg	?	NT	
3	Ages 10 59 yrs Sex: 8F 3M Race: 8W; 3C					
7	Bleeding Tendency: ++++	1				
	++	2				
	+ ±	5				
	0		1	-	1	
8	Tourniquet Test	1	9		-	
9	Bleeding Time: >10 min	1	9	-	-	
	Clotting Time					
10	glass >13 min		9	1†	-	†10 min
11	silicone >50 min	2	8			
12	Clot Retraction: ++ 0		10			
14	Platelet Count x 1000/mm ³ <100 (000)		10	-		†147 574
15	Platelet Tpln * <60	7	2		1	
16	Platelet Accel *: <40	7	1	1†		†50
17	Serum Serotonin* 50 or less	2†		-	8	†16 50
19	Plasma Thr C T : (incr)	6	3		1	
20	Serum Antithrombin (incr)	1 (sl)	7	2†	-	†decr (?)
21	Fibr (a) decr (<200 mg)	1	4			†120 mg
	(b) incr (>540 mg)	5†				
22	AHF*: <60		10	-		
23	PTC* <60		6		4	
25	Prothrombin Cons <90%	4	1	1†	4	†90%
26	Prothrombin Time* <70	2	8			
27	Prothrombin* <70	3	7			
28	Proconvertin*: <65	4	5	1†		†66
29	Proaccelerin* <60	1	9			
	N P N (or B U N) mg/100ml	10	0			†78 330

* percent of standard normal

TABLE LVII B Group VII (B): Platelet Problems in Uremics (1 out of 11 cases studied)

1 No 78		
2 Case	M Tu	
3 Age	13	
Sex	F	
Race	W	
4 Dates Examined	31 Jan '55	30 May 55
7 Bleeding Tendency: ++	++	+++
8 Tourniquet Test	neg	neg
9 Bleeding Time (minutes)	3	3½
Clotting Time:		
10 glass (minutes)	8	N T
11 silicone (minutes)	43	N T
12 Clot Retraction	+++	N T
14 Platelet Count $\times 100/\text{mm}^3$	346	340
15 Platelet Thromboplastin*	100	100
16 Platelet Accel * (?)	50	100
17 Serum Serotonin*	N T	N T
19 Plasma Thr Clotting Time	norm	N T
20 Serum Antithrombin	norm	N T
21 Fibr: (a) decr (<200 mg)		
(b) incr (>540 mg)	800	N T
22 AHF*	100	N T
23 PTC*	80	50
25 Prothrombin Cons %	95	N T
26 Prothrombin Time*	88	4
27 Prothrombin*	84	20
28 Proconvertin*	100	15
29 Proaccelerin*	100	22
30 Remarks	Died 31 May '55 from pyelonephritis (chr) in polycystic kidneys.	

* percent of standard normal

TABLE LVII A Group VII (A) Platelet Problems in Uremics (10 out of 11 cases studied)

1 2	Nos 68 77 Cases	FINDINGS				REMARKS
		Pos	Neg	?	N T	
3	Ages: 10 50 yrs Sex 8F; 3M Race: 8W; 3C					
7	Bleeding Tendency: +++++ ++ + ± 0	1 2 5 0				
8	Tourniquet Test	1	9	-		1
9	Bleeding Time: >10 min	1	9	-		
	Clotting Time:					
10	glass: >13 min		9	1†		†10 min
11	silicone: >50 min	2	8			
12	Clot Retraction: ++ 0		10			
14	Platelet Count x 1000/mm ³ <100 (1000)		10			†147 574
15	Platelet Tpln * <60	7	2	-	1	
16	Platelet Accel *; <40	7	1	1†	-	†50
17	Serum Serotonin*; 50 or less	2†			8	†16 50
19	Plasma Thr C T; (incr)	6	3	-	1	
20	Serum Antithrombin (incr)	1 (sl)	7	2†	-	†decr (?)
21	Fibr (a) decr (<200 mg) (b) incr (>540 mg)	1 5†	4		-	†120 mg
22	AHF*; <60		10			
23	PTC*; <60		6		4	
25	Prothrombin Cons <90"	4	1	1†	4	†907
26	Prothrombin Time*; <70	2	8			
27	Prothrombin*; <70	3	7			
28	Proconvertin*; <6%	4	5	1†	-	†66
29	Proaccelerin*; <60	1	9	-		
	N P N (or B U N) mg/100 ml	10	0		-	†78 330

* percent of standard normal

TABLE LIX. Prothrombin Consumption Tests (2 stage) on a Case of Liver Carcinoma (Metastasis) Treated with Radio Gold (Au^{198})

Day of therapy	-1	4	8	13	19	22
Plasma (u/ml)	510	470	313	340	445	184
1 hr serum (Resid pro %)	23	1	2	2	20	45
Platelets $\times 10^3$	243	280	235	420	158	48

TABLE LX Platelet Counts and Prothrombin Consumption in Three Dogs Injected with Radio Gold

DOG		I		II		III	
DOSE Au^{198} (mc/Kg)		20		10		5	
		Plat /mm ³	Pro Cons	Plat /mm ³	Pro Cons	Plat /mm ³	Pro Cons
Day:	0	354 500	96%	238 400		312 500	
	2	217 500	93%	765 000			
	4	242 500	95%			337 500	95%
	6	45 000	0	339 600	80%	372 500	89%
	8	22 500	14%	139 400	59%	135 000	97%
	10	(died 9th day)		219 000	73%		40%
	13			26 000	12%	77 500	33%
	18			13 100	37%	145 000	66%

TABLE LVIII. C p VIII. Plat 1 t P bl m i Co with Pl m Cl tti g Diff tti s

1	79	80	81	82	(83	84	85
2	B S	L F	R B	F M	E B	D H	C R
3	17	54	54	29	26	3	70
	M	M	M	M	F	F	M
	W	W	C	W	C	W	W
4	1954	1954	1954	1953	1953	1954	10 Jan 55
5	?	1 wk	4 y	?	?	3 y	28 Feb 55
6	0	0	0	0	0	0 (7)	?
7	+	+	+	+	+	+++	++
8	g	+	n g	eg	NT	+	NT
9	2 1/2	3	1 1/2	5	NT	5	NT
10	4 1/2	4 3/4	5	10 1/2	Ly 1	39	Ly 1
11	16	42	49	43	Lvs	4 h	Ly 1
12	+++	Ly 1	Lys	++	Ly 1	++++	Lys
13	0	+	+	0	+	0	Ly 1
14	31	89	90	9	128	240	+
15	0	15	43	50	NT	100	6
16	1	1	20	24	NT	5	1
17	150	100	100	100	NT	250	NT
18	NT	NT	NT	NT	NT	NT	NT
19	1	13	e 13	in	e 13	n m	ee 13
20	n m	NT	n m	e m	NT	m	e m
21	320	250	560	340	200	410	20
22	100	100	100	100	NT	100	100
23	20	40	20	50	20	90	100
24	NT	NT	NT	NT	NT	0	200y
25	90	95	95	93	NT	43	41
26	NT	34	53	20	23	1	7
27	41	25	30	44	43	113	78
28	43	2	25	57	59	108	79
29	45	40	40	40	80	0	10
30	LIVER DISORDERS					C g	C i m (P t
+	un exp'd to UK					1 h of	with circ latig
	Ja d, Ja d					AG	Fib in lysin
+	I m mbe ig of t t am					IN T bl LIL	

TABLE LXIII Effect of 59 Poth on the Activation by Stypven and Cephalin in the Presence of Various Factors Optimal Conditions for the Activation of Fibrinogen Clotting Time (min) with Incubation at 37°C and 1 min and 10 min periods (min) 26°C

TEST NO.	FACTOR DEFICIENCY	A.G. ADDED	STYPVEN ADDED	CEPHALIN ADDED	INCUBATION PERIOD (min) 1	optimal 1
1	AHF	+	+	+	5	24.2 23.2 (2)
2	PTC	+	+	+		34.5 22.6 (5)
3	Hag	+	+	+	29.7	17.2 (3)
4	Hg	+	+	+	24.6	23.7 (3)
5	VII	+	+	+	26.4	23.1 (2)
6	AcG		+	+	94.9	32.0 (4)
7	AHF	+		+	16.7	23.4 (11)
8	AHF	+	+		350.7	248.4 (10)
9	PTC	+	+		720	501 (10)
10	Hg	+	+		730	232.2 (20)
11	VII	+	+	203.5		76.5 (14)
12	Hg		+	+	175.2	121.3 (19)
13	(Hg+)		+	+	177.5	119.9 (19)
14	(AG+)	+	+	+	25.4	21 (2)

(Hg+ AG+ etc.)

TABLE LXI Thrombin Formation 2 stage tests Various Thromboplastic Agents with or without AHF Prothrombin and other factors constant

EXPT NO.	AGENT +Ca. AcG	INITIAL RATE		OPTIMUM PERIOD		THROMBIN YIELD (%)	
		AHF	AHF+	AHF	AHF+	AHF	AHF+
1	Tpln	fast	fast	short	short	100	99
2	Ceph	very slow	slow	long	short	66	95
3	Sbp		"		short*	10	73*
4	Eap				(long)*	2	9*
5	Plat				short	75*	92
6	Rbc				short	40	100
7	0	negligibl activation in the control				0	0

Technical details given in text Experiment 56

TABLE LXII Bioassay of Prothromboplastin by Stypven 2 stage method
Standard¹ = brain cephalin Unknown = Eap 26°C

TEST NO	AGENT TESTED	REL STR	FINAL CONC μg/ml	INCUBATION PERIOD:	
				1 min	optimal (min)
1	Ceph	100%	30.0	17.8	16.8 (2')
2		10%	3.0	34.0	20.4 (4)
3		8"	2.4	36.1	20.5 (6')
4		6"	1.8	46.0	21.1 (13')
5		4"	1.2	52.0	22.5 (15)
6		2%	0.6	77.8	25.3 (17)
7		1%	0.3	127.1	54.3 (20')
8	0 (control)	0	0	351	284" (10')
9	E p	1:1	400	69.8	23.4 (11)
10	Eap	1:2	200	82.5	27.9" (15')

APPENDIX III

BIBLIOGRAPHY

TABLE LXIV Experiment 59 IV Prothrombin Activation in Stuart deficient Eluate Optimal Ca and AcG Effects of adding Stypven cephalin thrombo plastin AHF and Stuart factor preparations (see text) Fibrinogen clotting times (sec) with incubate after 1 min and at optimum (min.) 26°C

TEST NO	AGENTS TESTED	FACTORS ADDED	INCUBATION PERIOD		THROMBIN YIELD (%)
			1 min	opt (min)	
1	Stypven		1800	1800 (7)	trace
2	Stypven	Stuart (A)	238 2"	216 1 (5')	trace
3	Stypven cepha.		59 3	27 4 (4)	52
4	Stypven cepha	Stuart (A)	22 4	16 8 (5')	100
5	Stypven cepha	Stuart (B)	24 7	19 9 (4)	89
6	Stypven tpin		64 3"	24 4 (7)	64
7	Stypven tpin	Stuart (A)	22 0	17 0 (5')	100
8	Tpin		184 8"	24 0 (11')	65
9	Tpin	Stuart (A)	30 8	17 4 (5)	98
10	Tpin	Stuart (B)	31 2	17 2 (4')	99
11	Ceph	AHF	315	26 3 (17)	55
12	Ceph	AHF Stuart (A)	73 6	16 0 (5')	104

TABLE LXV Various Prothromboplastins tested (2 stage) in Stypven activation of prothrombin (eluate)

TEST NO	AGENT TESTED	OPTIMAL TEST	
		CT	INC PERIOD
1	Ceph	23 0	2 min
2	Sbp	22 2	3 min
3	Plat	20 6"	4 min
4	Rbc	20 9	3 min
5	Eap	23 4	11 min
6	0	>240	

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